EFFECTS OF IN VIVO EXERCISE AND IN VITRO CONTRACTILE ACTIVITY ON THE REGULATION OF AS160, TBC1D1 AND GLUCOSE TRANSPORT IN RAT SKELETAL MUSCLE

by

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To Amanda

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On one cold morning in January of 2005, I flew in to Detroit Metro Airport for the first time. Dr. Greg Cartee was kind enough to pick me up early and introduced me to the city of Ann Arbor, the University of Michigan, and finally the lab that I now call home. He previously emailed me an itinerary of my visit, which meticulously detailed every minute of the day. In his office (which now is a part of CCRB 3040 classroom), we went over the exercise R01 grant draft and, in detail, focused on a newly identified protein called "ay-es-160." After returning to Boston, and prior to my decision on coming here I read a copy of that draft (with "Confidential" marked across the top) over and over again while I ran my last several Western blots. I was particularly impressed with its carefully crafted writing that I now know must have gone through at least a hundred drafts. For the last four and half years working with "GC", I've come to understand and appreciate his style in science. He is incredibly detail-oriented, and shows a great deal of mental endurance in thinking through even the smallest of projects. What we do in Muscle Biology Laboratory isn't really anything fancy per se, as glucose transport and protein phosphorylation have been studied for a while. "We don't have all the resources like the big-shots" he'd say, "so we have to have an edge over them in something else." Over the years I've come to understand that, as scientists, we've got to be able to ask the right questions to move forward, and fancy tools alone is not going to get us there. So what I would remember and cherish the most about the experience in this

lab isn't when I had to transfer muscles every minute for three hours straight, or when I had 42 blots going simultaneously. It's the weekly lab meetings in which we went over everyone's projects in detail, it's getting back yet another draft with marks and slashes all over, it's sitting in his office for 2 hours with dry-erase markers in our hands, and it's the " let's have another practice presentation" few hours before our seminar. I thank you for that, Greg.

So last year after I submitted my 91 page dissertation proposal to my committee members for approval, I overheard Dr. Chuck Burant say to Greg "What was that THING Katsu brought to my office last week?" I thank all my committee members, Drs. Charles "Chuck" Burant, Jeff Horowitz and Jiandie Lin for carefully reviewing my progress. I also thank Drs. Katarina Borer, Pete Bodary and Marvin Boluyt for their support and advice.

When a certain former PhD graduate from Kinesiology defended his dissertation, Dr. Ed Arias noticed that this particular student was choked with emotions after he acknowledged Ed's name. Ed, you and I know that all that emotion wasn't directed towards you, but I have to say that you may deserve that from me somewhat. You're the nicest and friendliest lab manager that I know, and takes little credit for being the second mentor that you've been to many past and current lab members. You have been around from the very beginning of my training, you have been a great help and a friend.

In 2005 when I joined Muscle Biology Laboratory, there were only four of us, Greg, Ed, Dr. Taku Hamada and me. In those early years, both Tak and I endured the onslaught of grant experiments (that are now R01 AG-010026 and DK-071771) everyday with little experience in the art of glucose transport (and Ed at the dissection table for all

of them). Now the grants are funded and more people in the lab are here to help out. While the intensity has worn off slightly, I know the time is coming for renewal and I'm glad to let Tak know that I won't be here to experience it again.

David Blair joined our lab in 2006 as a cynical junior undergraduate student immediately after Tak left us. He was convinced that the greatness was ahead of him (probably is) and graciously allowed us to help him in his cause. He talked and did everything so fast that probably the best thing Greg taught him was to slow down (and he did a little bit). He'll probably be the head of some elite medical school 30 years from now. The following year, our lab interviewed several candidates for a postdoc position but we would have nothing less than Dr. Naveen Sharma, a bright young star from Case Western. It didn't matter what we were studying, he was going to convince us that studying heart was what was most important. He routinely cites Sharma et al. 2005, Sharma et al. 2006 and Sharma et al. 2007 to get us back at where all our research focus should be; the heart. Also I've never met another Indian-Filipino-Canadian that is more fluent in the language of pop-culture that no another American is more American than you. Thanks for all the goodness in and out of science.

Amanda and I welcomed Andrew to our family on May 10, 2007, and from then on life has been a continuous juggling act. It may have been insane for both of us to continue with our Ph.D. programs without wanting to rely heavily on babysitter/daycare. It's difficult to say whether it's been worthwhile, but I am a better person because of it. One particular difficulty was that I couldn't be at two different places at the same time. I needed to give 100% at home because not doing house chores like cleaning (which obviously needed done every day over again and again) meant that there would be less

time for Amanda to work. Conversely, I needed to be extra attentive at work because staying late was no longer an option. Part of my dilemma is that I haven't been willing to give up either of the end of spectrum: 1) be a good Dad that spends time with kids and try to do as much at home, and 2) be a productive and successful scientist who has passion and patience in the lab. So leaving work at 5:30pm was hard both ways; I wanted to leave early so I could help Amanda get more work done, but I wanted to stay longer because I wanted to get more work done. One thing I know is that I wouldn't change this for the world. I love being a father, and the kids need me. I don't think life was ever meant to be easy, but it sure has been fruitful.

I need to thank Maria Cholakova who watched our two kids occasionally. I am also deeply grateful to Dr. Jeff Fessler, who has been the best mentor that Amanda could have. It's true when I say that you were a God-sent to us. You have been extremely kind, understanding, flexible and encouraging. If it wasn't for your support, we would have stopped trying to pursue our studies long time ago.

Shortly after the birth of Andrew, George Schweitzer and Carlos Castorena joined our lab as new Ph.D. students. In many aspects, the summer of 2007 was a pivotal period in my doctoral training. The presence of these two guys totally changed the dynamics in the lab. I probably should be lot more grateful for the support that these guys provided because if I think about it carefully, I couldn't have asked for better guys to join the lab. George has babysat our kids on several occasions and Andrew loves George. Andrew's first four people words were Dad, Mom, Jesus and George. We are also coauthors on many of our papers. In lab, he was always available to help out by swimming rats, transferring muscles or talking science. He's definitely the nicest guy in the lab. Carlos,

is a born scientist, but more importantly for me, he also is a father. There is nothing like having a coworker whose life circumstance is similar. In some ways, he has it harder because his kids are closer in age, he has to teach classes and he already had a child when he started his Ph.D. I know it must be really hard for him (and for Katherine), but he never shows it. That's been a great source of strength to me. He always hates when people use family issues as excuses for things not getting done. To some people that may seem arrogant, but I know that he says that because he never allows himself to use his personal life as an excuse.

I have never seen Jim MacKrell frown. This is true as he always seems to have a smile on his face. So having him join us at the beginning of 2008 was another boost in morale, although I also feel like sometimes I don't really understand him at all. He's little bit like Dave, in that they both have a flash of genius. He seems to be always on the go and his single fiber stuffs is often times over the top of my head. Like him, Donel Sequea (another Physiology transplant) always seems to be on the run, attending seminars throughout the week. I don't know if it's because Donel's an MSTP student, but he seems to know everyone on campus. He's always saying "hi" and hugging people on the street and catching up. He's inherited my Akti-1/2 project so perhaps some of those data, so riddled with caveats that we decided not to submit it for publication, may see the light some day. Abhi Bhat's meticulous knowledge in seemingly every single sports on the planet (including cricket) was definitely much appreciated. It was an irreplaceable asset to have another person fluent enough in the language of professional soccer that I could have a meaningful exchange of information. Steve Ho picked up right where Abhi left off, both in and out of science. In science, Steve Ho has been

meticulously good about tracking the calorie restriction study, just as Abhi had been. Out of science, Steve Ho appears to share Abhi's love for sports. One thing that may differentiate these guys is that Steve Ho has a python in his apartment. Anketse Kassa recently joined our lab and has been highly influential to us all: we haven't had a permanent female worker in this lab since 2005. The first week she was here she cleaned the microwave and organized all the plastic utensils in our office. We each hopefully have become a bit more considerate for our workplace because of it. I also acknowledge Ankush Bansal, Julianne Wilke and Nikki Herlich for their assistance in lab. I also thank many of the Kinesiology staff, especially to Leona Cranford, Charlene Ruloff, Sandra Wiley and Carrie Braun for their support.

During the first three years in Ann Arbor, we spent lot of our leisure time with David and Caroline Garcia, until he graduated and left for Manhattan to work for Credit Suisse in the middle of 2008. David has been absolutely outrageous and hilarious. There is a lot to be said about how great life can be in difficult times when you have a good friend. I still remember when he got a yellow card for cursing and getting in a fight during the IM indoor soccer game, and when he ate a whole quart of bubble gum ice cream. The life at Credit Suisse seems quite stressful now, but I hope you get your groove back soon and chillax a little. I also thank our other friends including Jared and Sarah Lyle, Zach George, David McOmber, Edgar and Tiffany Lee, Landon Carrel and Warren Tate for their friendship.

If I had to pick one person in my life that I look up to, and want to be like, it would be my mom. Growing up, my siblings and I always felt that she had too big of a personality for us to handle. She always sang the loudest at church. She would hug, kiss

and hold our teenage hands in public. During Undōkai, she'd be the only mom that would be cheering our names with flags in front of everyone. One summer she MADE all of us kids (4 of us under age 11) go watch Flamenco in Barcelona (which started at 11pm) and to all our shame, decided to join the dancers on the stage. I realize now what a unique gift she's been to me. She's always lived her life to the fullest. It's uncommon to see a person who knows exactly what she wants, and goes and does it without hesitation. It's not even that she dreams big, but rather it's that she so intensely wants things to happen, nothing stops her. She taught us to have faith. She loved us with all her heart. My dad was the quiet and patient one, who always kept cool and understood the child in us better. It was because of his love for soccer, that Kiyo and I are both clinically addicted to it. I still recall some of the most fun times in my life when we all used to kick balls at the Shimane University Track. He earned three doctorates, yet he never boasted and always helped with our elementary maths homework. Now he's transitioned well to a provider of intellectual discussions, in history, medicine, sociology and philosophy. Kiyo and I have been the closest of siblings, as we shared our rooms almost throughout our childhood. We did everything together, that I don't remember not having you around at all. I am super excited for you and Hiroko (for your soon-to-be-born baby and PhDs) and hope that whatever your next step in life will be, that you'll always be happy. Tomo, it's great to see your happy family with Masami and Takuto, too. It's awesome to know that our kids will have cousins close to each other's ages. Congratulations on your upcoming graduation and I have confidence, however stressful the unforeseeable future may be, that you'll earn a wonderful life. Izumi, the last but most definitely not least of my siblings, I cannot thank you enough for your sacrifice for the last few years, doing the

unthinkable of putting your academics on hold to take care of mom's health. I know you had other reasons to be with family now, but nonetheless without you I wouldn't have felt peace being so far away from home. I must tell you that I believe in you strongly, and since your were little you were indeed special. And know that my respect for you is not measured by the scale of outward accomplishments, but of your heart. I also thank my other family members, especially Takako Funai, Fumio Funai, Tomiko Oshima, Noriko Narita and Ikuko Kubori who I consider contributed significantly toward where I am today.

Andrew and Seth, daddy has been away a whole lot to work, but every day I miss you two there (George misses you, too, Andrew). Daddy loves you two very much. Daddy learned so many things at work, but also learned a whole bunch more at home from you two, too. You two taught me that loving someone means that you spend time with them. You taught me that loving someone means that it makes you want to help selflessly. Having you two around teaches me how much my parents must have loved me and cared for me. Most importantly you two taught me how to love someone unconditionally. It's been so much fun to be your father.

Amanda, I am going to try to express my feelings in words (rather than saying "words cannot express" because, in my opinion, that's a cop-out for not trying hard enough). I've asked of you a lot, especially for the last two and half years. I have not been very flexible in my schedule. I treated my work as 9-5, as if yours wasn't. I put a lot of unfair expectations on your shoulders, partly because of lack of my decisiveness. Yet you've worked harder with more focus. If it weren't for having children, you probably would have graduated a semester or two ago. Even then, you've never

compromised or dismissed a responsibility to be a mother, because you've never been short-sighted. Every day seeing you work hard was a reminder that I needed to do the same. I don't think I was able to help nearly as much as I wanted, but occasionally being able to be there for you brought great joy to me. I can't believe that we've been together for over 8 years now. It seems like we've always been swamped with things to do, yet over the memories of difficult times, I cherish the plenty of fun that we had together. Looking back over the last four and half years, none of the progress (if I ever truly made one) would have come if it weren't for you being by my side. Of the many things that we have accomplished together, this dissertation may be the least of it, but I hope that you accept my gratitude as a dedication, of all the work that has gone into this dissertation, to you.

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ABSTRACT

A single bout of exercise leads to an increase in insulin-independent and insulin-dependent increase in glucose transport (GT). Phosphorylation of two members of the TBC1 (tre-2/USP6, BUB2, cdc16) domain family of proteins, Akt substrate of 160 kDa (AS160, also known as TBC1D4) and TBC1D1, has been suggested to regulate the increase in GT. The purpose of the studies in this dissertation was to provide insights into the roles that AS160 or TBC1D1 phosphorylation play in the insulin-independent and insulin-dependent increases in GT after in vivo exercise or in vitro contraction using rat epitrochlearis muscle. Immediately after in vivo exercise or in vitro contraction, the insulin-independent GT was elevated concomitant with increases in the phosphorylation of AS160 and TBC1D1. However, in experiments using pharmacological inhibitors, wortmannin (inhibits phosphatidylinositol 3-kinase) or Compound C (inhibits AMP-activated protein kinase), the increased AS160 phosphorylation after in vitro contraction was uncoupled from increased GT, whereas TBC1D1 phosphorylation and insulin-independent GT consistently tracked together. Furthermore, TBC1D1 phosphorylation and GT returned to resting values 3 h post-exercise, whereas AS160 phosphorylation remained elevated. In contrast, the prolonged increase in AS160 phosphorylation, but not TBC1D1 phosphorylation, at 3 and 27 h after in vivo exercise coincided with enhanced insulin-stimulated GT. Additionally, AS160 phosphorylation and insulin-stimulated GT both reversed to resting levels in rats fed carbohydrate-rich chow for 3 h post-exercise.

In another set of experiments, doubling the amount of exercise (from 1 to 2 h) or electrical stimulation in serum (from 5 to 10 tetani) did not further elevate insulin-stimulated GT. In contrast, the combination of prior exercise (2 h) and electrical stimulation (10 tetani) had an additive effect on the subsequent increase in insulin-stimulated GT, suggesting that exercise and electrical stimulation may amplify insulin sensitivity through distinct mechanisms. These results suggest that: 1) TBC1D1 phosphorylation, but not AS160 phosphorylation, may be important for insulin-independent increase in skeletal muscle GT immediately after in vivo exercise or in vitro contraction; and 2) AS160 phosphorylation, but not TBC1D1 phosphorylation, may be important for insulin-dependent increase in skeletal muscle GT several hours after in vivo exercise, but not after in vitro contraction.

CHAPTER I

INTRODUCTION

The molecular mechanisms by which contractile activity or exercise leads to increases in insulin-independent and insulin-dependent glucose transport in skeletal muscle are not fully understood (7, 21, 22, 24, 34). Two members of the TBC1 (tre-2/USP6, BUB2, cdc16) domain family of proteins, Akt substrate of 160kDa (AS160, also known as TBC1D4) and TBC1D1, have been suggested to potentially regulate the increase in GLUT4 translocation that is essential for increasing glucose transport (7, 8, 25, 29, 33, 37, 38). AS160 and TBC1D1 each contain a Rab GTPase-activating protein (RabGAP) domain that appears to have an inhibitory effect toward the Rab proteins that are implicated for GLUT4 vesicle trafficking (25, 33). Phosphorylation of AS160 or TBC1D1 is believed to inactivate RabGAP activity, which in turn may allow activation of Rab proteins and initiate GLUT4 translocation (8, 25, 33, 38). In skeletal muscle, insulin or contraction can result in increased phosphorylation of AS160 and TBC1D1 (1, 2, 5, 26, 45), suggesting that these proteins may also be involved in insulin- or contraction-stimulated increase in glucose transport in this tissue (5, 7, 27, 37). The primary goals of the research described in this dissertation were: 1) to elucidate mechanisms whereby in vivo exercise or in vitro contractile activity regulates the

phosphorylation of skeletal muscle AS160 and TBC1D1; and 2) to gain insights into the roles that AS160 and TBC1D1 phosphorylation may play in the increased skeletal muscle glucose transport after exercise or contractile activity.

A single bout of exercise or in vitro contractile activity results in an increase in insulin-independent glucose transport (also known as contraction-stimulated glucose transport) during and immediately after exercise (22, 24, 31, 34, 50). Study 1 and 2 investigated the regulation of AS160 or TBC1D1 phosphorylation immediately after in vitro contraction.

Study 1: Contraction-stimulated Glucose Transport in Rat Skeletal Muscle is Sustained despite Reversal of Increased PAS-phosphorylation of AS160 and TBC1D1

In a purified enzyme assay (cell-free condition), AMP-activated protein kinase (AMPK) or Akt can each phosphorylate AS160 or TBC1D1 (9, 16, 45). However, the kinase(s) that phosphorylate AS160 or TBC1D1 during muscle contraction remains unclear. Furthermore, activation of Akt is not essential for contraction-stimulated glucose transport (28, 35, 49), whereas AMPK has been implicated for mediating contraction-stimulated glucose transport (20, 48). Consistent with the idea that AMPK, but not Akt, is important for contraction's effect on increasing skeletal muscle glucose transport, previous studies demonstrated transient contraction-stimulated activation of Akt (36), but sustained activation of AMPK (39, 41, 43) or glucose transport (30). No studies had reported the time-course of AS160 or TBC1D1 phosphorylation in response to in vitro contraction. Therefore, Study 1 evaluated the time-courses for contraction-

stimulated phosphorylation of AS160 and TBC1D1, along with these kinases and glucose transport.

Study 2: Inhibition of Contraction-stimulated AMPK Inhibits Contraction-stimulated Increases in PAS-TBC1D1 and Glucose Transport without Altering PAS-AS160 in Rat Skeletal Muscle

To more directly assess the relationship between AMPK or Akt and AS160 or TBC1D1, Study 2 used pharmacological inhibitors that prevented the contraction-stimulated activation of either AMPK (compound C) or Akt (wortmannin) and examined their effects on contraction-stimulated phosphorylation of AS160 or TBC1D1. Furthermore, this study determined whether the attenuation of the contraction-stimulated increase in AS160 or TBC1D1 phosphorylation, secondary to inhibition of upstream kinases, was accompanied by reduced contraction-stimulated glucose transport.

In addition to the insulin-independent increase in glucose transport during and immediately after in vivo exercise or in vitro contraction, a single bout of exercise or in vitro contractile activity in serum leads to a subsequent increase in insulin-stimulated glucose transport (the insulin-dependent effect of exercise, also known as increase in insulin sensitivity) (14, 17, 19, 21, 32). Study 3 and Study 4 investigated a possibility that AS160 or TBC1D1 phosphorylation may play a role in enhanced insulin-stimulated glucose transport several hours after in vivo exercise or in vitro contraction.

Study 3: Increased AS160 Phosphorylation, but Not TBC1D1 Phosphorylation, with Increased Post-exercise Insulin Sensitivity in Rat Skeletal Muscle

The post-exercise increase in insulin-stimulated glucose transport is mediated by enhanced insulin-stimulated GLUT4 translocation to the cell-surface membranes (19). However, the cellular mechanism(s) leading to this event are unknown (21). Proximal insulin signaling steps such as insulin binding (3, 4, 52), insulin receptor tyrosine kinase activity (40, 42, 46), insulin receptor tyrosine phosphorylation (19, 23, 42, 47), insulin receptor substrate tyrosine phosphorylation (19, 23, 51), insulin receptor substrate associated phosphatidylinositil 3-kinase activity (40, 47), and Akt serine phosphorylation (2, 11, 18, 40, 46) are not enhanced in insulin-stimulated muscles that have undergone prior exercise. Recently, several studies reported that, in contrast to these upstream signaling events, AS160 phosphorylation remain elevated for many hours after a bout of in vivo exercise (2, 12, 39, 44). The level of TBC1D1 phosphorylation post-exercise had not been previously reported. In Study 3, the possibility that a prolonged increase in AS160 or TBC1D1 phosphorylation may play a role in post-exercise increase in insulin-stimulated glucose transport was explored. In three different post-exercise conditions that were known to result in either enhanced or reversed insulin-stimulated glucose transport, AS160 or TBC1D1 phosphorylation were measured to test whether each consistently accompanied the changes in insulin-stimulated glucose transport.

Study 4: In Vivo Exercise Followed by In Vitro Contraction Additively Elevates Subsequent Insulin-stimulated Glucose Transport by Rat Skeletal Muscle

In vitro contraction of isolated skeletal muscle in serum-free buffer induces an increase in insulin-independent glucose transport comparable to the increase after in vivo exercise. In contrast, when isolated skeletal muscle is stimulated to contract, a subsequent increase in insulin-stimulated glucose transport is observed only when serum was present during the in vitro contraction (6, 13). Therefore, it has been hypothesized that a serum factor is necessary for a post-exercise increase in insulin-stimulated glucose transport (10, 13, 15, 21). In Study 4, to probe the possibility that AS160 or TBC1D1 phosphorylation may play a role in the post-contraction increase in insulin-stimulated glucose transport, isolated skeletal muscles were stimulated to contract in the presence or absence of serum to examine whether changes in AS160 or TBC1D1 consistently tracked with changes in insulin-stimulated glucose transport. Furthermore, the effect of in vivo exercise, with and without subsequent electrically stimulated contraction, on insulin-stimulated glucose transport was determined to see if these two stimuli (in vivo exercise and in vitro contraction) would have additive effects on insulin sensitivity.

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CHAPTER II

REVIEW OF LITERATURE

Significance of Skeletal Muscle Glucose Transport

Type 2 diabetes mellitus (non-insulin dependent diabetes mellitus, NIDDM) accounts for 90 to 95% of all cases of diabetes, and approximately 6% of the adult population in Western society suffers from the disease (107, 135). Insulin resistance by skeletal muscle is a primary defect in the progression of Type 2 diabetes (39, 114). Furthermore, insulin resistance is associated with poor health even in people who do not become diabetic (48, 62, 96). Skeletal muscle accounts for up to 85% of insulin-stimulated glucose clearance (40), and glucose transport is considered a rate-limiting step in muscle glucose metabolism (180). In addition to insulin-stimulation, physical exercise can positively modulate glucose homeostasis due to enhanced insulin-independent glucose transport and elevated insulin action in the working muscles. The effects of exercise to increase skeletal muscle glucose transport and insulin sensitivity may help explain, at least in part, the strong epidemiological evidence that regular exercise prevents or delays the onset of Type 2 diabetes (90, 150).

In addition to these health benefits, the effects of exercise on skeletal muscle glucose transport are important for healthy individuals with regard to energy provided from blood glucose during exercise and the rapid replacement of muscle glycogen after

exercise. Repeated bouts of vigorous exercise in athletes or people with physically demanding occupations can deplete muscle glycogen and the increased skeletal muscle glucose transport is an essential component for replenishing muscle glycogen. Providing blood glucose or replenishing muscle glycogen also likely has survival benefits in animals living in the wild.

Insulin and Exercise

Insulin and exercise co-regulate skeletal muscle glucose transport by increasing the number of cell surface GLUT4 glucose transporters (58, 155). Multiple lines of evidence suggest that the effects of insulin and exercise on glucose transport are distinct: 1) insulin and exercise stimulate separate pools of GLUT4 vesicles to be translocated to the plasma membrane (36, 43); 2) the effects of a maximally effective concentration of insulin plus exercise on skeletal muscle glucose transport or GLUT4 translocation are additive (37, 101); 3) insulin, but not exercise/contraction, activates insulin-receptor substrate (IRS)-associated phosphatidylinositol 3-kinase (PI3K) (57); 4) PI3K inhibitor wortmannin blocks the effect of insulin on glucose transport without affecting the effect of contraction in isolated skeletal muscle (100, 170); 5) muscles from Zucker rats that are insulin resistant have normal contraction-stimulated glucose transport (9, 16); 6) muscles from transgenic mice that are null for the insulin receptor or Akt2 have reduced insulin-stimulated glucose transport but have normal contraction-stimulated glucose transport (103, 127, 165); 7) exercise/contraction, but not insulin, stimulates AMP-activated protein kinase (AMPK) and Ca^{2+}/calmodulin dependent kinase II (CaMKII) (67, 168); 8) mice with kinase-dead AMPK that have reduced contraction-stimulated glucose

transport have normal insulin-stimulated glucose transport (108). Although many studies have elucidated possible pathways by which insulin or exercise regulates glucose transport, precise mechanisms that lead to an increase in cell surface GLUT4 are not fully understood.

Effects of Exercise on Skeletal Muscle Glucose Transport

The first published evidence for the insulin-like effect of exercise on glucose transport was from horse masseter muscle in which a chewing action was found to induce a difference in arterio-venous glucose concentration (27). In 1957, Helmreich and Cori more directly investigated this phenomenon by studying isolated rat skeletal muscle, and found that muscle contraction increased pentose accumulation (69).

A single bout of in vivo exercise can increase glucose transport in skeletal muscle during and immediately after exercise (73, 84, 125). This increase in exercise-induced glucose transport can occur in the absence of insulin (115, 156) and therefore is also known as insulin-independent glucose transport (or direct effect of exercise). The insulin-independent effect on glucose transport begins to reverse shortly after the cessation of contractile activity (155) and most of this effect is lost by 1-3 hr post-exercise (173). Subsequently, a much more persistent increase in sensitivity to insulin-stimulated glucose transport occurs in skeletal muscle, lasting 3-48 hr (55, 61, 64, 119). This effect is known as the increase in insulin-dependent glucose transport (or indirect effect of exercise).

The Insulin Signaling Pathway

Insulin increases glucose transport by increasing GLUT4 (a major glucose transporter in skeletal muscle) translocation to cell surface membranes (Fig. 2.1) (10, 98, 170). Insulin-stimulated GLUT4 translocation is dependent on insulin signaling initiated by the binding of insulin to its receptor on cell surface, causing autophosphorylation on multiple tyrosine residues in cytosolic domain (19, 142, 160, 176). The tyrosine phosphorylation on the insulin receptor (IR) subsequently recruits multiple docking proteins that activate several signaling cascades, including IRS proteins, which in turn also become tyrosine phosphorylated as a result of the increased to insulin receptor tyrosine kinase activity (109, 131, 141, 159). Tyrosine phosphorylated IRS proteins recruit multiple proteins including PI3K (20). PI3K binding to IRS induces a conformational change in PI3K that results in activation of its kinase domain (20). PI3K phosphorylates phosphatidylinositol-(3,4)-bisphosphate (PIP2) in the phospholipid bilayer to produce phosphatidylinositol-(3,4,5)-trisphosphate (PIP3) (151, 153). Proteins that contain pleckstrin homology (PH) domains, including phosphoinositide-dependent kinases-1 (PDK1) and Akt, are recruited to PIP3 lipid rafts (3, 99). PDK1 activates Akt by phosphorylating Thr^{308} on its activation loop (1, 2) and phosphorylation on Ser^{473} by mammalian target of rapamycin complex-2 (mTORC2) stabilizes its activation (133). Activation of Akt is reportedly sufficient to increase glucose transport (91, 112). Among many substrates that Akt can phosphorylate, Akt substrate of 160 kDa (AS160, also known as TBC1D4) has been recently linked to insulin-stimulated GLUT4 translocation and glucose transport (88, 132).

Role of AS160 in Insulin-stimulated Glucose Transport

AS160 contains a Rab GTPase-activating protein (RabGAP) domain that can regulate Rab-GTP formation (Fig. 2.2, top) (88, 105, 132). A family of Rab proteins is involved in membrane vesicle trafficking, and these proteins can exist in an active GTP-bound form or in an inactive GDP-bound form (41, 137, 178). Activity of Rab proteins is regulated by three classes of proteins including: 1) guanine nucleotide exchange factor (GEF) that converts inactive GDP-bound form to its active GTP-bound form; 2) GAP that causes GTP hydrolysis resulting in GDP formation; and 3) guanosine nucleotide dissociation inhibitors (GDI) that prevents Rab proteins from being turned on and retain its GDP-bound form. The balance among these three classes of proteins is essential in optimizing the GTP-to-GDP ratio of Rab proteins (139). Unphosphorylated AS160 has an active RabGAP domain that promotes GTP hydrolysis, thus inhibiting Rab activation (105). Phosphorylation of AS160 seems to turn off its RabGAP activity, leading to decreased GTP hydrolysis of Rab proteins (105, 132). Consequently the balance among GEF, GAP and GDI shifts towards an increase in GTP formation and induces Rab-mediated GLUT4 translocation (Fig. 2.3).

Evidence that phosphorylation of AS160 regulates GLUT4 translocation in 3T3-L1 adipocytes was initially shown by Gus Lienhard's group (132). 3T3-L1 adipocytes expressing AS160 with alanine mutations on four insulin-responsive phospho-Ser/Thr residues (called the 4P mutant) had a reduced insulin-stimulated GLUT4 translocation to the cell surface. RabGAP domains contain an arginine residue that is critical for their activity, and mutation of this residue to lysine abolishes RabGAP activity (132, 162). Insulin-stimulated GLUT4 translocation in cells expressing mutated AS160 with inactive

RabGAP (R973K mutant, R/K) was not different from that in wild-type cells. Importantly, cells expressing double mutant AS160 (both R/K and 4P) did not exhibit the inhibitory effect that the 4P mutation alone has on insulin-stimulated GLUT4 translocation. Together, these observations in 3T3-L1 adipocytes suggested that: 1) the active RabGAP domain of AS160 inhibits GLUT4 translocation; 2) phosphorylation of AS160 induces insulin-mediated inactivation of RabGAP domain; and 3) phosphorylation-mediated inactivation of the RabGAP domain of AS160 is essential for the full insulin-stimulated GLUT4 translocation.

Tim McGraw's group used Total Internal Reflection Fluorescence Microscopy (TIRF) to assess the cell surface dynamic of GLUT4 and found that insulin-stimulated GLUT4 translocation is significantly reduced in 3T3-L1 adipocytes with AS160-4P overexpression, without significantly affecting basal GLUT4 translocation (177). Importantly, they found that cells expressing the AS160-4P mutant had a residual increase in insulin-stimulated GLUT4 translocation. Further analyses indicated that while insulin regulates both exocytosis and endocytosis of GLUT4 vesicles, only exocytosis is regulated by AS160. They subsequently studied the effect of siRNA-mediated knockdown of AS160 (46) and found that basal GLUT4 translocation was elevated in cells that did not express AS160, supporting the idea that AS160 negatively regulates GLUT4 translocation.

To summarize, studies using 3T3-L1 adipocytes suggested that AS160 inhibits glucose transport by suppressing GLUT4 exocytosis (but not endocytosis) through AS160's RabGAP activity. Insulin-stimulated phosphorylation of AS160 appears to inactivate RabGAP, resulting in increased GLUT4 exocytosis. A variety of experimental

approaches, including AS160 manipulation (overexpression, knockdown and inhibitors) and measures related for glucose transport (cell surface GLUT4, TIRF measurements of GLUT4 movement and 2-deoxyglucose uptake), have provided substantial evidence for AS160 playing a key role in insulin-stimulated glucose transport in 3T3-L1 adipocytes.

Role of AS160 Phosphorylation in Insulin-stimulated Glucose Transport in Skeletal Muscle

Arias et al. (4) and Bruss et al. (17) subsequently reported in rat skeletal muscle that insulin results in an increase in AS160 phosphorylation detected by phospho-Akt substrate (PAS) antibody. The commercially available PAS antibody was designed to recognize multiple Akt phosphorylation motif peptide sequences (RXRXXpT/S). The time-course and dose-response for AS160 phosphorylation were consistent with the ability of physiological concentrations of insulin to rapidly increase glucose transport by isolated skeletal muscle. The incubation of muscles with the PI3K inhibitor wortmannin eliminated the insulin-stimulated increase in Akt Ser^{473} phosphorylation and AS160 phosphorylation, indicating that the effect of insulin on AS160 in skeletal muscle, as in adipocytes, is PI3K- and presumably Akt-dependent. Using primary human skeletal muscle cells, Juleen Zierath's group reported that IRS1 and Akt2 siRNA-mediated knockdown, but not IRS2 or Akt1, resulted in elimination of insulin-stimulated AS160 phosphorylation (14). The idea that Akt2 is important in insulin-stimulated AS160 phosphorylation is supported by the lack of increased insulin-stimulated AS160 phosphorylation in Akt2 null mice (93). Taken together, insulin-stimulated phosphorylation of AS160 in skeletal muscle seems to occur through the same pathway

as adipocytes, suggesting that insulin-stimulated glucose transport in skeletal muscle may also be regulated byAS160 phosphorylation.

In collaboration with Lienhard's group, Laurie Goodyear's group studied the effect of AS160 mutants on insulin-stimulated glucose uptake in mouse skeletal muscle (94). They electroporated vectors for the 4P and/or R/K mutants of AS160 to investigate effects of AS160 mutation on insulin-stimulated glucose uptake in mouse tibialis anterior muscle. Similar to the findings in adipocytes, expression of the 4P mutant of AS160 resulted in a decreased insulin-stimulated glucose uptake and the 4P-R/K double mutation rescued this effect. These results suggested that, in skeletal muscle as in adipocytes, the insulin-stimulated phosphorylation of AS160 is important for the effect of insulin on glucose uptake in skeletal muscle, and this effect requires a functional RabGAP domain on AS160.

To summarize, findings in intact skeletal muscle are consistent with the idea that insulin-stimulated phosphorylation of AS160 is important for the regulation of increase in glucose transport. However, current understanding of the role of AS160 in insulin-stimulated glucose transport in skeletal muscle relies heavily on findings in 3T3-L1 adipocytes.

Cell Localization of GLUT4 Vesicles and AS160

In both 3T3-L1 adipocytes and L6 myoblasts, GLUT4 continuously cycles between intracellular pools and the cell surface (44, 59). GLUT4 is largely retained intracellularly through idle cycling between endosomal pools, and in both cultured muscle and adipose cells the major depot is in the perinuclear area. In both muscle and

adipose cells, GLUT4 traffic to the cell surface likely involves several steps: 1) GLUT4 vesicle release from retention in specialized pool(s); 2) mobilization of GLUT4 vesicles towards the cell surface; 3) GLUT4 vesicle tethering, docking and fusion with the plasma membrane (157).

In 3T3-L1 adipocytes, separation of subcellular compartments using sucrose gradient fractionation revealed that in the basal state, vesicular compartments that contain GLUT4 also contain AS160 (97, 105), suggesting that AS160 is associated with GLUT4 vesicles in the unstimulated state. Immunoprecitation of insulin-regulated aminopeptidase (IRAP) resulted in AS160 co-immunoprecitation, suggesting that AS160 may be bound to the GLUT4 vesicles through IRAP (113). One study reported that insulin treatment induced AS160 to dissociate from GLUT4 vesicles (97), but another study did not detect any insulin effect on the amount of AS160 associated with IRAP (113). No studies have addressed in skeletal muscle if AS160 subcellular localization is regulated with respect to GLUT4 vesicles.

Role of 14-3-3 Proteins in AS160-dependent Action on GLUT4 Vesicles

In addition to IRAP and GLUT4 vesicles, AS160 has been suggested to be associated with 14-3-3 proteins (116). 14-3-3 is a class of proteins whose function is facilitated by interacting with phospho-serine or phospho-threonine residues in a variety of other proteins. The interaction of 14-3-3 with target proteins has been shown to encode a variety of functions including subcellular redistribution, altered protein conformation, protection from proteolysis, impaired interaction with other proteins and scaffolding (15).

In 3T3-L1 adipocytes, 14-3-3 association to AS160 appears to increase in response to insulin stimulation (116). Interestingly, cells expressing either the 4P or the T642A (threonine substituted for alanine at the 642 residue of AS160) mutant of AS160 did not interact with 14-3-3, whereas cells expressing wild-type AS160 did bind 14-3-3, indicating that 14-3-3 may bind to AS160 on phosphorylated Thr642 site (56, 116). In addition, attenuated insulin-stimulated GLUT4 translocation in cells that overexpressed AS160-4P was rescued by restoration of 14-3-3 binding to AS160, suggesting that 14-3-3 binding to AS160 is important for insulin-stimulated glucose transport (116). Consistent with these findings in cells, 14-3-3 binding capacity of AS160 is greater in human skeletal muscle biopsy samples taken immediately after a hyperinsulinemic-euglycemic clamp compared to pre-clamp biopsy samples, supporting the idea that 14-3-3 binding to AS160 may also be functionally important in insulin-stimulated skeletal muscle (76, 77).

Calmodulin Binding Domain of AS160 in Insulin-stimulated Glucose Transport

Lienhard's group first recognized that AS160 contains a calmodulin binding domain located near C-terminus (88, 132). The calmodulin binding domain of AS160 can bind to calmodulin in the presence of Ca^{2+}, and calmodulin is co-immunoprecipitated with AS160 (87). However, in 3T3-L1 adipocytes, overexpression of AS160 with a mutated calmodulin binding domain that prevents calmodulin binding to AS160 did not alter basal or insulin-stimulated GLUT4 translocation indicating that a functional calmodulin binding domain of AS160 is not essential for the insulin-stimulated increase in glucose transport in fat cells (87).

AS160-independent Mechanisms Involved in Insulin-stimulated Glucose Transport

Although a great deal of evidence suggests that AS160 is likely involved in insulin-stimulated glucose transport, AS160 is likely not the only downstream factor affecting GLUT4 translocation. As described previously, AS160-dependent mechanisms are not important for insulin-stimulated inhibition of GLUT4 endocytosis (177). The absence of increased basal GLUT4 at the cell surface (or increased glucose transport) in cells expressing the AS160 R/K mutant suggests that other steps also regulate GLUT4 vesicles (94, 132, 177).

Based on results from a study that used TIRF to systematically assess the docking rate and the fusion rate of GLUT4 vesicles in 3T3-L1 adipocytes (7, 85), insulin apparently caused increases in both of these steps. However, overexpression of AS160-4P mutant caused a complete inhibition of insulin-stimulated increase in the docking rate and appeared to have no effect on fusion rate. These results suggested that AS160 is involved only in the docking step of the insulin-stimulated GLUT4 translocation, and that additional mechanisms are involved in insulin-stimulated increase in GLUT4 vesicle fusion to the cell membrane. Similarly, a study that used confocal fluorescence microscopy on rounded-up L6 myoblasts (117) showed that overexpression of AS160-4P mutant only inhibited the docking step of insulin-stimulated regulation of GLUT4 vesicles and had no effect on GLUT4 tethering. Together, these studies suggest that AS160-dependent mechanisms are essential but not sufficient for the full insulin-stimulated increase in glucose transport.

Role of TBC1D1 in Insulin-stimulated Glucose Transport

Recently, Lienhard's group (121) found that TBC1D1, a closely related protein to AS160 (which is also known as TBC1D4), also becomes phosphorylated with insulin stimulation in 3T3-L1 adipocytes and therefore could potentially influence GLUT4 translocation. TBC1D1 is a protein of roughly the same apparent molecular mass as AS160 that is 47% identical and 67% similar to AS160 over its entire length (Fig. 2.2, bottom). The GAP domain of TBC1D1 is 79% identical and 91% similar to that of AS160 and regulates activity of the same Rab proteins (2A, 8a, 8b, 10 and 14) as AS160.

Similar to their approach in the earlier study with overexpression of AS160 mutant in 3T3-L1 adipocytes (132), they tested the effects of TBC1D1 mutant overexpression on insulin-stimulated GLUT4 translocation (121). The TBC1D1 mutants studied included: 1) TA (phosphorylation site Thr^{596} mutated to Ala) mutant of TBC1D1 (comparable to T642A mutant of AS160); 2) arginine to lysine (R/K) mutation of the GAP domain that eliminates GAP activity of TBC1D1 (comparable to R/K mutant of AS160); or 3) TA-R/K double mutant (comparable to 4P-R/K double mutant in AS160). Similar to the observations in AS160 mutant overexpression experiments (132), insulin-stimulated GLUT4 translocation in TBC1D1 mutant overexpressed cells was: 1) decreased in cells that overexpressed TA mutant of TBC1D1; 2) unchanged in cells that overexpressed R/K mutant compared to cells with empty vector overexpression; or 3) unchanged in cells that overexpressed TA-R/K mutant compared to cells with empty vector overexpression. However, one key difference between observations from TBC1D1 and AS160 overexpression studies was that although the overexpression of wild-type AS160 did not result in the inhibition of insulin-stimulated GLUT4

translocation, overexpression of wild-type TBC1D1 resulted in the inhibition of insulin-stimulated GLUT4 translocation. These results suggested that the decrease in insulin-stimulated GLUT4 translocation observed in cells that overexpressed TA mutant of TBC1D1 was not due to the threonine to alanine mutation per se, but was likely due to the overexpressed TBC1D1 protein in these cells. They interpreted this difference to indicate that either: 1) phosphorylation of TBC1D1 by insulin is incomplete; and/or 2) phosphorylation of TBC1D1 by insulin is not sufficient to inhibit its GAP activity. TBC1D1 has fewer Akt phosphorylation sites than AS160 and has an AMPK phosphorylation site that is absent in AS160 (121). Therefore it seemed possible that AMPK, and not Akt, may be the crucial kinase that regulates the GAP activity of TBC1D1.

Chavez et al. (28) subsequently performed TBC1D1 knockdown in 3T3-L1 adipocytes and found no effect on insulin-stimulated GLUT4 translocation. In 3T3-L1 adipocytes, TBC1D1 protein expression is only ~5% as abundant as AS160, and therefore endogenous TBC1D1 may not be important for insulin-stimulated GLUT4 translocation in these cells. As described above, TBC1D1 overexpression resulted in a significant reduction (~90%) of insulin-stimulated GLUT4 translocation (28, 121). AMPK activator 5'-aminoimidazole-4-carboxamide ribonucleoside (AICAR) also did not increase GLUT4 translocation in 3T3-L1 adipocytes overexpressing wild-type TBC1D1. Interestingly, in TBC1D1 overexpressing 3T3-L1 adipocytes, simultaneous incubation with both insulin and AICAR (which activates AMPK) significantly attenuated (~70%) the reduction in GLUT4 translocation caused by TBC1D1 overexpression (28). These results suggested: 1) TBC1D1 can regulate GLUT4 translocation in 3T3-L1 adipocytes

when expressed in a much higher amount than usual; and 2) AMPK signaling may be necessary for TBC1D1-dependent regulation of GLUT4 translocation. TBC1D1 protein is much more highly expressed in skeletal muscle than white adipose tissue (143), and AICAR stimulates glucose transport by AMPK-dependent pathway in skeletal muscle (51). Therefore these results suggested that TBC1D1 may be a component in the signal transduction pathway leading to AMPK-stimulated GLUT4 translocation in muscle.

Ishikura et al. (79) examined the effects of AS160 or TBC1D1 knockdown on cell-surface GLUT4 in basal and insulin-stimulated conditions in L6 myotubes which express relatively high levels of both AS160 and TBC1D1. As expected, AS160 or TBC1D1 each resulted in an increase in cell-surface GLUT4 at basal state compared to non-related siRNA control, consistent with the idea that AS160 or TBC1D1 each functions as a stop signal for basal GLUT4 translocation. Silencing AS160 resulted in unchanged insulin-induced GLUT4 translocation (insulin-dependent net gain above basal values for cell surface GLUT4) compared to non-related siRNA control, compatible with the idea that insulin-stimulated phosphorylation of AS160 relieves the stop signal that AS160 has on GLUT4 translocation. However, silencing TBC1D1 resulted in greater insulin-induced GLUT4 translocation compared to non-related siRNA control, suggesting that TBC1D1 participation in GLUT4 traffic is not regulated by insulin. In other words, TBC1D1 knockdown allowed insulin to induce a greater amount of GLUT4 vesicle translocation than in cells that express TBC1D1. These results does not eliminate the possibility that TBC1D1 can regulate an insulin-independent increase in glucose transport (e.g., with contraction).

Carol MacKintosh's group found that either Akt or AMPK can phosphorylate purified TBC1D1 on several sites in a cell-free kinase assay, but only phosphorylation by AMPK resulted in increased 14-3-3 binding (29). In L6 myotubes, AICAR, but not insulin, increased 14-3-3 binding to immunoprecipitated TBC1D1 (29), consistent with the finding that insulin-stimulated phosphorylation of TBC1D1 is not sufficient to increase GLUT4 translocation (28, 121). In addition, immunocytochemical analysis of L6 cells revealed that TBC1D1 was co-localized with GLUT4 in unstimulated cells (29). However, they did not detect TBC1D1 dissociation from GLUT4 in cells that were incubated in insulin or A-769662 (AMPK activator) (29).

Goodyear's group showed in mouse skeletal muscle that either insulin or AICAR can stimulate TBC1D1 phosphorylation detected using the PAS antibody, supporting the findings in cell lines (143). Using mass spectrometric analysis of TBC1D1, they suggested that, in mouse skeletal muscle, AICAR caused greater overall phosphorylation of TBC1D1 sites compared to insulin, indicating that AMPK may be a robust regulator of TBC1D1 phosphorylation. Further studies are necessary to elucidate the role of TBC1D1-mediated regulation of glucose transport in skeletal muscle.

To summarize, studies in 3T3-L1 adipocytes indicate that endogenous TBC1D1 may not be important for inducing the insulin-stimulated glucose transport because: 1) these cells contain only ~5% of TBC1D1 protein compared to AS160; and 2) overexpression of wild-type TBC1D1 inhibit insulin-stimulated GLUT4 translocation. In cell-free kinase assay, purified Akt is not sufficient to increase 14-3-3 binding capacity of AS160. Results from studies in L6 myotubes, which express higher levels of TBC1D1, indicate that TBC1D1 may be important for the regulation of insulin-independent

GLUT4 translocation. Several lines of evidence suggest that AMPK may regulate TBC1D1 phosphorylation. It would be important to determine if TBC1D1 plays a role in the AMPK-dependent increase in GLUT4 translocation that occurs with skeletal muscle contraction.

Exercise/Contraction-stimulated Glucose Transport

As discussed above, multiple reports provide evidence that there are distinct mechanisms for the stimulation of glucose transport by exercise/contraction versus insulin. Considerable evidence suggests that both AMP-activated protein kinase (AMPK) and increased cytosolic calcium are involved in contraction-induced, but not insulin-stimulated, increase in GLUT4 translocation (Fig. 2.4) (75, 108, 168).

Numerous studies have investigated the roles of that AMPK as a critical signaling molecule for the regulation of multiple metabolic, protein synthetic and transcriptional processes that are altered by contraction in skeletal muscle. AMPK is a heterotrimer complex that consists of α, β and γ subunits (65). The α-subunit of AMPK exhibits catalytic activity (106), where as the β-subunit acts as a scaffold for the binding of α- and γ-subunits (166), and the γ-subunit has been proposed to be involved in binding of AMP (30). AMPK is activated by an increase in the AMP-to-ATP and creatine-to-phosphocreatine ratios via a complex mechanism that involves allosteric modification and phosphorylation (65).

Contraction results in the activation of AMPK via AMP binding and an increase in phosphorylation of AMPK on Thr172 by several AMPK kinases (51). AICAR (which is converted into AMP analog ZMP upon cell-entry) stimulation is sufficient to increase

glucose transport in skeletal muscle (104). AICAR-stimulated glucose transport in skeletal muscle is not inhibited by the PI3K inhibitor wortmannin. Furthermore, the increase in glucose transport with the combination of maximally effective AICAR plus maximally effective insulin concentration is partially additive, whereas there is no additive effect on glucose transport with the combination of AICAR plus contraction (67). Transgenic mice that overexpress a dominant inhibitory mutant of AMPK had a 30-40% reduced contraction-stimulated glucose transport in isolated EDL muscle compared to wild type controls (108) supporting the idea that AMPK contributes to the contraction-stimulated increase in glucose transport. In contrast, both AMPK α1 and α2 knockout mice exhibit normal contraction-stimulated glucose transport, despite completely inhibited AICAR-stimulated glucose transport (86). It is possible that the lack of inhibition is attributed to compensatory mechanisms in these models, perhaps related to the multiple AMPK isoforms, but it also raises important questions regarding the essential role of AMPK in regulating contraction-stimulated glucose transport. One possibility is that other signaling pathways such as calcium signaling may compensate for the lack of AMPK function. Alternatively, the large family of AMPK-related proteins could function redundantly in signaling for increased glucose transport (84).

John Holloszy and Hiro Narahara provided the first evidence that calcium may be a signaling messenger that leads to the contraction-stimulated increase in glucose transport in frog sartorius muscle (75). They found that an increase in cytosolic calcium induced by caffeine caused an increase in muscle glucose transport. Because the caffeine-induced increase in cytosolic calcium occurs independently of membrane depolarization, this suggested that contraction-stimulated glucose transport is stimulated

by a membrane-depolarization independent mechanism. Holloszy's group subsequently published a series of papers that further supported the idea that intracellular calcium contributes to the increase in glucose transport by contraction. In addition to caffeine, other agents such as W-7 that result in increased cytosolic calcium concentration also induce the increased glucose transport, and this stimulation can be inhibited by dantrolene which inhibits calcium release from sarcoplasmic reticulum (171, 172).

Intracellular calcium interacts with the binding protein calmodulin (33), and three signaling pathways from this calcium-calmodulin complex have been proposed to regulate contraction-stimulated glucose transport. CaMKII, the dominant CaMK isoform of skeletal muscle that is activated through binding of the calcium-calmodulin complex, has been implicated in contraction-stimulated glucose transport because both caffeine- and contraction-stimulated glucose transport can be inhibited with CaMKII inhibitors KN62 and KN93 (167, 168). However, the downstream target of CaMKII that is related to glucose transport has not been identified. Calmodulin also interacts with nitric oxide synthase (NOS) that activates NOS signaling. Some studies suggested that the inhibition of contraction-stimulated activation of NOS (with NOS inhibitors) may reduce contraction-stimulated glucose transport (122, 140), although others did not see this effect (47, 71, 126). In addition to binding to CaMKII and NOS, calmodulin binding to AS160 has been implicated in contraction-stimulated glucose transport (92). As discussed above, AS160 contains a functional calmodulin binding domain that is not involved in insulin-stimulated glucose transport (87). The overexpression of the AS160 mutant with a disrupted calmodulin-binding domain in mouse tibialis anterior muscle had a decreased contraction-stimulated glucose transport, indicating that calmodulin-binding

to AS160 may play an important role in contraction-stimulated glucose transport (92). The role of the TBC1D1 calmodulin-binding domain in contraction-stimulated glucose transport has not been investigated.

Calcium can also directly activate conventional and novel protein kinase C (PKC) which has also been suggested to be involved in contraction-stimulated glucose transport. Downregulation of PKC by long-term phorbol ester treatment (35), inhibition of PKC using polymyxin B (70, 175) or calphostin C (66) has each been associated with decreases in contraction-stimulated glucose transport.

Wright et al (168), showed that co-incubation of rat epitrochlearis muscle in AICAR and caffeine resulted in an additive increase in glucose transport, suggesting that AMPK and calcium increase glucose transport through independent mechanisms. Some reports suggest that intracellular calcium can increase AMPK activity by CaMK kinase (CaMKK)-dependent phosphorylation of AMPK on Thr^{172}, so it is also possible that a portion of the calcium signaling effect is mediated through AMPK signaling (81, 82, 118, 134). However, there is currently no evidence that suggest that CaMKII signaling or calmodulin binding to AS160 can converge downstream with AMPK signaling that is related to increasing glucose transport, so a portion of calcium signaling is apparently mediated in an AMPK-independent manner.

According to some reports (17, 127, 128, 130), contraction can also result in the activation of Akt, an important protein involved in insulin-stimulated glucose transport (18, 34, 72, 91, 161). The mechanism for the effect of contraction on Akt is uncertain. As mentioned above, contraction-induced Akt activation can be inhibited by wortmannin or LY294002, consistent with a PI3K-dependent process (130). However, contraction

does not increase the activity of class I_A PI3K (57, 129, 130) or class II PI3K (136). In this context, Sakamoto et al. (2002) suggested that contraction instead activates Akt through class I_B PI3K, an enzyme that is activated by G-protein coupled receptors rather than tyrosine kinase-coupled receptors (151, 152).

Although multiple lines of evidence suggest that Akt plays an essential role in insulin-stimulated glucose transport (18, 34, 72, 91, 112, 161), Akt is not likely to be involved in contraction-stimulated glucose transport. Wortmannin, a PI3K inhibitor that results in complete inhibition of contraction-stimulated activation of Akt, has no effect on contraction-stimulated glucose transport (100, 170). Skeletal muscles from Akt2 knockout mice, which have substantially reduced insulin-stimulated glucose transport (103), have a normal contraction-stimulated glucose transport (127).

To summarize, it appears that both AMPK and calcium signaling play essential roles in the contraction-stimulated increase in glucose transport. Although some evidence suggests that AMPK and calcium signaling may partially converge, the additivity of these pathways suggests that these are largely independent mechanisms by which each regulates glucose transport. Downstream targets of AMPK and calcium signaling leading to increased glucose transport have yet to be fully elucidated.

Role of AS160 in Contraction-stimulated Glucose Transport

Bruss et al. (17) first showed that in vitro contraction of isolated rat epitrochlearis muscle results in an increased PAS-AS160 (measured in samples after AS160 immunoprecipitation). Subsequently this finding was supported in mouse extensor digitorum longus (EDL) that was stimulated to contract either in vitro or in situ (93), and

in skeletal muscle sampled immediately after in vivo endurance exercise by humans (42), rats (5) and mice (93).

Mechanisms for Contraction-stimulated Phosphorylation of AS160

The precise mechanism by which contractile activity results in increased PAS-AS160 is unclear. The contraction-stimulated increases in Akt Ser^{473} phosphorylation and PAS-AS160 were fully inhibited when isolated muscles were stimulated to contract in the presence of the PI3K inhibitor wortmannin (17), indicating that the contraction-stimulated increase in PAS-AS160 was PI3K-dependent (and presumably Akt-dependent) in rat epitrochlearis muscle. Importantly, because wortmannin does not affect contraction-stimulated glucose transport (100, 170), these results seem to strongly suggest that increased PAS-AS160 is not essential for contraction-stimulated glucose transport.

Bruss et al. (17) also found that incubation of isolated rat epitrochlearis muscle with the AMPK-activator AICAR caused a small, but significant increase in PAS-AS160 (measured in sample that had undergone AS160 immunoprecipitation). Because AICAR did not affect Akt phosphorylation, this 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 (149). As described above, contraction leads to the activation of AMPK (17, 51), so it seemed possible that AMPK could PAS-phosphorylate AS160 during contraction. However this appeared not to be the case in rat epitrochlearis muscle with the contraction protocol that was used for this study as

indicated by the complete elimination of contraction-stimulated increase in PAS-AS160 in wortmannin treated muscles (17) even though wortmannin does not inhibit AMPK (8, 169).

AICAR may also lead to increased AS160 phosphorylation in mouse skeletal muscle (93, 149). Goodyear's group (93) investigated the effect of wortmannin on contraction-stimulated increase in AS160 phosphorylation. Instead of immunoprecipitating muscle samples with AS160 (PAS-AS160), they used anti-PAS immunoblot and measured a band at ~160kD (PAS-160) or used anti-phospho-AS160 Thr^{642} (pThrAS160) as an indication of AS160 phosphorylation. They found that wortmannin appeared to only partially inhibit contraction-stimulated increase in PAS-160 and pThrAS160. Furthermore, tibialis anterior muscle from Akt2-null mice compared to wild-type controls had a roughly similar contraction-stimulated increase in PAS-160 or pThrAS160 despite the lack of a contraction-induced increase in Akt Thr^{308} phosphorylation in the null mice. Therefore, in mouse skeletal muscle, a substantial portion of the contraction-stimulated increase in PAS-160 and pThrAS160 apparently occurs by an Akt-independent manner.

Studies using isolated EDL muscle from mice with genetically modified AMPK found that: 1) α2-inactive AMPK overexpression resulted in a reduction of contraction-stimulated increases in PAS-160 or pThrAS160 (93); 2) kinase-dead AMPK overexpression resulted in a suppression of contraction-stimulated increase in PAS-160 (149); and 3) AMPK α2 knockout resulted in a suppression of contraction-stimulated increase in PAS-160 (149) . Taken together, these observations provide evidence that AS160 can be a substrate of AMPK, and that AMPK can account for a portion of

contraction-stimulated increase in AS160 phosphorylation, at least in mouse skeletal muscle. It is notable that none of these studies immunoprecipitated samples using anti-AS160 prior to immunoblotting with anti-PAS or anti-pThrAS160. Because AMPK is thought to be involved in contraction-stimulated glucose transport, and AS160 in insulin-stimulated glucose transport, determining whether contraction-stimulated glucose transport occurs through AMPK-dependent phosphorylation of AS160 would be important.

It is unclear what factors account for the differences in findings in contraction by isolated muscles from rats (in which Akt appeared to account for most phosphorylation of AS160) and mice (in which AMPK appeared to contribute to phosphorylation of AS160). The studies differed with regard to the muscles studied (rat epitrochlearis vs. mouse EDL), but both the rat epitrochlearis and mouse EDL are predominantly fast-twitch muscles. Another difference was that rat samples were immunoprecipitated with anti-AS160 prior to immunoblotting with anti-PAS (PAS-AS160) (17), whereas mouse muscles lysates were immunoblotted without prior immunoprecipitation (PAS-160, pThrAS160) (93, 149), although a subsequent report found that contraction-stimulated increase in PAS-160 (without prior AS160 immunoprecipitation) was also completely wortmannin inhibitable in rat epitrochlearis (25). It is possible that the mouse muscles studied without prior AS160 immunoprecipitation contained signals from other proteins that have immunoreactivity towards anti-PAS or anti-pThrAS160. It is possible that phosphorylation of TBC1D1, a paralog of AS160 that has a similar molecular weight and contains similar peptide sequence, might have been included in the signals from the

mouse muscles without prior AS160 immunoprecipitation although this possibility has yet to be tested.

To summarize, the mechanism whereby AS160 becomes phosphorylated in response to a contractile activity remains unclear. Evidence suggests that either Akt or AMPK can phosphorylate AS160, but the possible activation of compensatory mechanism in genetically modified mice and the possibility that signals from TBC1D1 may have been included in measurement of PAS-160 or pThrAS160 in mouse muscle make the interpretation difficult. Further studies must address this by: 1) using experimental methods that can acutely inhibit Akt or AMPK instead of long-term inhibition that occur in transgenic or knockout models; and 2) differentiating AS160 and TBC1D1 by prior immunoprecipitation.

Role of AS160 Phosphorylation in Contraction-stimulated Glucose Transport in Skeletal Muscle

Kramer et al. (94) studied the effect of overexpressing wild type or mutant AS160 in mouse tibialis anterior muscle to assess the role of AS160 phosphorylation on contraction-stimulated glucose transport. Similar to their observations on insulin-stimulated glucose uptake, the contraction-stimulated increase in glucose uptake was: 1) reduced with overexpression of 4P mutant AS160; and 2) R/K mutation rescued the inhibitory effect of 4P mutation (4P-R/K double mutation). These data suggest that AS160 phosphorylation can regulate contraction-stimulated glucose transport. However, there were some observations that suggested that different mechanisms are involved in AS160-dependent regulations of insulin- and contraction-stimulated glucose transport.

While overexpression of wild-type AS160 had no effect on insulin-stimulated glucose uptake, it resulted in a small, but significantly reduced contraction-stimulated increase in glucose uptake compared to muscles transfected with empty vector. Overexpression of R/K or 4P-R/K double mutant of AS160 did not alter insulin-stimulated glucose uptake, but contraction-stimulated glucose uptake in these muscles was slightly, but significantly greater than those transfected with empty vector. It is unclear if the unexpected discrepancies were attributable to artifacts of genetic overexpression. Regardless, the interpretation of how these data relate to the role that AS160 phosphorylation plays in contraction-stimulated glucose transport should be made cautiously.

In addition to these results that suggest a complex relationship between contraction-stimulated AS160 phosphorylation and glucose transport, there are other data that uncouple increased AS160 phosphorylation from contraction-stimulated glucose transport. As described earlier, wortmannin, a PI3K inhibitor that has no effect on contraction-stimulated glucose transport (100, 170), can completely inhibit contraction-stimulated PAS-AS160 (17). One caveat is that there is evidence that anti-PAS does not interact equally to all of the Akt phosphomotifs of AS160. Recombinant Akt1 can phosphorylate recombinant wild-type AS160 in vitro, as detected with anti-PAS (88). When the experiment was repeated using a mutant-AS160 with alanine substituting for Ser^{588}, there was no diminution of the PAS-signal; however, when alanine was substituted for Thr^{642}, the PAS-signal was nearly eliminated, suggesting that PAS-reacts strongly to the Akt phosphomotif including Thr^{642}, but not Ser^{588}. Therefore, it is possible that AMPK-dependent phosphorylation of AS160 on sites undetectable by PAS may be intact in muscles incubated in wortmannin, and that phosphorylation of AS160 on

these wortmannin-resistant sites is sufficient for GLUT4 translocation and glucose transport to be elevated.

There is additional evidence that argues against the role of AS160 phosphorylation in contraction-induced glucose transport. AMPKα2-null mice that have reduced contraction-stimulated AS160 phosphorylation (PAS-160 and pThrAS160) have normal contraction-stimulated increase in glucose transport (86). However, it is possible important to recognize that genetic modifications in AMPK might have induced compensatory adaptations. Another study in mouse soleus muscle indicated that low frequency twitch stimulation that results in increased glucose transport did not result in an increase in PAS-160, providing further evidence that increased PAS-AS160 is not essential for contraction-stimulated glucose transport (83). In addition, the post-exercise increase in PAS-AS160 can remain elevated above resting values long after contraction-stimulated glucose transport has reversed (5). This indicated that not only is PAS-AS160 unnecessary for glucose transport, but it also is not sufficient to increase glucose transport.

To summarize, results using overexpression of mutant AS160 in mouse tibialis anterior muscle suggest that AS160 phosphorylation can regulate contraction-stimulated glucose transport. However, 1) reduced contraction-stimulated increase in glucose transport in muscles with wild-type AS160 overexpression, and 2) greater contraction-stimulated increase in glucose transport in muscles with AS160 R/K mutant or 4P-R/K double mutant overexpression (compared to vector overexpression in both cases), suggested that different mechanisms are involved in the role that AS160 plays for the regulation of insulin- and contraction-stimulated glucose transport. It also seems possible that because of the structural similarity between AS160 and TBC1D1, overexpression of

AS160 may have resulted in the subcellular displacement of the endogenous TBC1D1, influencing GLUT4 vesicles that are associated with both AS160 and TBC1D1. In addition, several studies reported that contraction-stimulated increases in PAS-AS160 and glucose transport can be uncoupled, suggesting that PAS-AS160 may not be essential for the contraction-stimulated increase in glucose transport. Further studies are necessary to differentiate the role that AS160 and TBC1D1 phosphorylation may play on contraction-stimulated glucose transport.

Calmodulin Binding Domain of AS160 in Contraction-stimulated Glucose Transport

The presence of a functional calmodulin binding domain in AS160 (87) suggests that AS160 may be a downstream target of calcium signaling that triggers contraction-stimulated glucose transport. Kramer et al. (92) studied the effect of overexpressing calmodulin binding domain (CBD)-mutant AS160 on contraction-stimulated glucose uptake in mouse tibialis anterior muscle. Similar to the results in 3T3-L1 adipocytes (87), overexpression of CBD-mutant AS160 had no effect on insulin-stimulated glucose uptake. Consistent with their previous report (94), overexpression of wild-type AS160 resulted in a small but significant reduction in contraction-stimulated increase in glucose uptake. Overexpression of CBD-mutant AS160 further reduced the increase in contraction-stimulated glucose uptake, providing indirect evidence that the CBD of AS160 may play a role in the calcium-dependent increase in contraction-stimulated glucose transport. However, similar to their previous observation on 4P-R/K double mutant of AS160 (94), overexpression of CBD-R/K double mutant of AS160 also resulted in greater contraction-stimulated glucose uptake when compared to the muscles that were transfected with

empty vector, emphasizing again that these results with protein overexpression must be cautiously interpreted. Importantly, the contraction-stimulated increase in glucose uptake was not further diminished in muscles that overexpressed CBD and 4P double mutation of AS160, compared to muscles that overexpressed CBD or 4P mutant alone. These results suggested the increases in calmodulin binding to AS160 and phosphorylation of AS160 are both essential for the contraction-stimulated increase in glucose transport (92).

Role of TBC1D1 in Contraction-stimulated Glucose Transport

The possibility that TBC1D1 may play a role in the contraction-stimulated increase in glucose transport has not been tested. As discussed above, skeletal muscle contains much greater amounts of TBC1D1 protein compared to adipose tissue (143). In addition, purified AMPK is sufficient to increase 14-3-3 binding to immunoprecipitated TBC1D1, but not AS160, in cell-free assay (29). TBC1D1 becomes PAS-phosphorylated in response to either AICAR or contraction stimulation (143). It seems reasonable to suspect that contraction-stimulated PAS-TBC1D1 may play a role in contraction-stimulated glucose transport. TBC1D1 also contains a calmodulin binding domain (121) that may regulate contraction-stimulated glucose transport. Further studies are necessary to understand what role, if any, that TBC1D1 plays in contraction-stimulated glucose transport. As mentioned above, previous quantification of PAS-160 and/or pThrAS160 without prior AS160 immunoprecipitation in mouse skeletal muscle (93, 149) may have included signals from both PAS-AS160 and PAS-TBC1D1. Because AS160 and TBC1D1 are structurally similar, it is possible that the overexpression of one of the

paralogs results in subcellular displacement of the other, affecting GLUT4 vesicles that are potentially associated with both AS160 and TBC1D1.

Post-exercise Increase in Insulin-stimulated Glucose Transport

Acute exercise can markedly enhance the subsequent insulin-stimulated glucose transport (55, 61, 119). This increase becomes apparent 1 to 3 hours post-exercise when most of the direct effect of exercise (insulin-independent glucose transport) is lost (173) and can last 3 to 48 hours (24, 26). The post-exercise increase in insulin-stimulated glucose transport at ~3 hours after acute exercise is attributable to increased insulin-stimulated GLUT4 cell surface localization after exercise (64) without increased total GLUT4 abundance (23).

However, this increase in insulin-stimulated glucose transport after exercise seems to occur in the absence of enhanced upstream insulin signaling (Fig. 5) (21). Most studies showed that prior exercise has no effect on insulin signaling steps such as: 1) IR binding (11-13, 181); 2) IR tyrosine phosphorylation (32, 64, 78, 147, 165); 3) IR tyrosine kinase activity (145, 147, 163, 165); 4) IRS tyrosine phosphorylation (64, 78, 164, 165, 179); 5) IRS-PI3K association (38, 49, 145, 164, 165); 6) Akt serine phosphorylation (5, 32, 49, 63, 145, 163); and 7) Akt threonine phosphorylation (63).

In contrast to unchanged insulin signaling at proximal steps, Arias et al. (5) showed that PAS-AS160 is greater in insulin-stimulated muscles from exercised (4 hours post-exercise) rats compared with sedentary controls (Fig. 5). However, this was not because AS160 phosphorylation induced by insulin-stimulation was increased, but because the basal PAS-AS160 in post-exercise muscles remained elevated 4 hours post-

exercise. In other words, prior exercise did not increase insulin-sensitivity for PAS-AS160, rather PAS-AS160 remained above resting 4 hours post-exercise independent of insulin. This observation is also supported by results in humans that showed that PAS-160 can remain elevated for 2 to 14 hours post-exercise without elevated insulin (50, 138).

Unphosphorylated AS160 is believed to restrain GLUT4 vesicles from moving toward the cell surface under basal conditions, and insulin-mediated AS160 phosphorylation appears to relieve this restraint. In this context, the presistent increase in AS160 phosphorylation after exercise might also be predicted to attenuate this restraint. Why, then, is the increased AS160 phosphorylation several hours after exercise not accompanied by a large and persistent increase in glucose transport in the absence of insulin? As discussed above, increased AS160 phosphorylation appears to be necessary, but not sufficient for insulin-stimulated glucose transport. Studies in both 3T3-L1 adipocytes (7) and L6 myoblasts (117) suggest that AS160 phosphorylation is required for insulin-stimulated docking of GLUT4 vesicles, but that other mechanisms are necessary for a fusion of GLUT4 proteins to the cell surface membrane. If additional insulin-stimulated processes are required for increasing GLUT4 translocation, it would explain why sustained increase in basal PAS-AS160 is not sufficient to cause an increase in basal glucose transport at ~4 hours post-exercise (5). It seems reasonable to suspect that the putative additional regulatory step(s) also becomes activated upon insulin stimulation, and in conjunction with greater PAS-AS160 (as a result of prior exercise), a greater amount of GLUT4 becomes fused with cell surface membranes. Although this idea has yet to be tested, it is intriguing because no other insulin signaling step has been shown to become amplified in insulin-stimulated muscle after exercise.

The post-exercise increase in insulin sensitivity can last for 3-48 hours, with the time course for reversal depending on post-exercise carbohydrate consumption (26, 61, 64). A study of rat epitrochlearis muscle indicated that the post-exercise increase in insulin sensitivity can be maintained for up to 48 hours if rats were not refed with carbohydrate (i.e., either not refed anything or fed only a high-fat, carbohydrate-free diet) after exercise (26). Although low muscle glycogen has been suggested to contribute to increased insulin-stimulated glucose transport after exercise (182), rats that were fed a high-fat and carbohydrate-free diet for 48 hours post-exercise or rats that were fed high-carbohydrate chow for 3 hours post-exercise exhibited increased insulin-stimulated glucose transport despite their post-exercise glycogen concentration returning to values similar to unexercised controls. These results indicate that reduced muscle glycogen concentration is not essential at the time of the increase in post-exercise increase in insulin-stimulated glucose transport. Also in support of this idea, prior incubation of rat epitrochlearis muscles in AICAR (which leads to AMPK activation) and serum (required for post-exercise increase in insulin-stimulated glucose transport as described below) resulted in a subsequent increase in insulin-stimulated glucose transport compared to muscles that were not incubated in AICAR (49). Because AICAR and serum did not cause a decrease in muscle glycogen concentration, this suggests that a decrease in muscle glycogen concentration may also not be required for the increase in insulin-stimulated glucose transport that is observed after exercise (which also activates AMPK).

Although the post-exercise increase in muscle insulin sensitivity persisted even when glycogen concentration was restored to pre-exercise values, it was found that in muscles from rats fed a high carbohydrate diet for 18 hours after exercise, which resulted

in accumulation of glycogen to 50% greater than usual concentration (i.e., glycogen supercompensation), the enhanced insulin-stimulated glucose transport was completely reversed (26). Thus, it is possible that glycogen supercompensation, or some related process, may be involved in the mechanism for reversal of the post-exercise increase in insulin sensitivity in muscle.

In contrast to what happens after in vivo exercise, in vitro contractile activity by isolated muscle in serum-free buffer does not result in a subsequent increase in insulin-stimulated glucose transport (24). Gao et al. (52) demonstrated that a factor found in serum [protein(s) with molecular weight greater than 10kD] must be present for muscle contraction to induce a subsequent increase in insulin sensitivity. In vitro contraction of isolated rat epitrochlearis in the presence of serum, followed by 3.5-hour incubation in the absence of serum, resulted in greater insulin-stimulated glucose transport compared with muscles treated identically except for the absence of serum during the contraction. The identity of the serum factor is still unknown.

To summarize, the mechanism whereby prior exercise results in increased insulin-stimulated glucose transport is unknown. This increase in insulin-stimulated glucose transport appears to occur in the absence of enhanced upstream insulin-signaling that have been studied with the possible exception of AS160 phosphorylation. Further studies are necessary to more thoroughly test if the sustained increase in AS160 phosphorylation is consistently found with the post-exercise increase in insulin-stimulated glucose transport.

Rationale for Research Models Used in This Thesis

Rats were used for the experiments in this thesis. There is a large body of literature from the last fifty years that describes both contraction-stimulated glucose transport and the post-exercise increase in insulin-stimulated glucose transport in rat skeletal muscle. Characterization of the contraction-stimulated glucose transport includes, but is not limited to: 1) insulin-independent increase in contraction-stimulated GLUT4 translocation (43, 53); 2) time-course of glucose transport with sustained contraction (111); 3) reversal of increase in glucose transport after the cessation of contractile activity (155, 173); 4) contraction intensity dose-response of increase in glucose transport (110); and 5) signaling cascade thought to be involved in contraction-stimulated glucose transport (17, 75, 168, 171, 172). The post-exercise increase in insulin-stimulated glucose transport is also well characterized in terms of, but not limited to: 1) the post-exercise increase in insulin-stimulated GLUT4 translocation (64); 2) time-course of post-exercise increase in insulin-stimulated glucose transport (26, 119); 3) insulin dose-response for the post-exercise increase in insulin-stimulated glucose transport (55, 61, 80, 119); 4) effects of post-exercise feeding on increased insulin-stimulated glucose transport (26, 174); 5) factors necessary for post-contraction increase in insulin-stimulated glucose transport in isolated skeletal muscle (24, 45, 49, 52); 6) lack of increased insulin signaling post-exercise (22, 49, 64, 147, 181); and 7) increased PAS-AS160 in skeletal muscle post-exercise (5).

The epitrochlearis muscle were used in the research in this thesis. The rat epitrochlearis muscle is a thin muscle arising from the tendon of insertion of m. latissimus dorsi and inserting into the medial epicondyle of the humerus. Its function is

to assist extension of the antebrachium (31). It is innervated by the ulnar nerve and is supplied with blood via the muscular branch of a. profunda brachii (60). In animals weighing 112-132 g, the muscle is 21-24 fibers and 0.7-0.8 mm in thickness (154). This diffusion distance has been estimated to be short enough to maintain adequate tissue oxygenation (95), making it appropriate for in vitro muscle incubation and glucose transport measurement. In support of this, long-term incubation (30 hours) had no effect on ATP, phosphocreatine or lactate levels, demonstrating that the muscles maintained their energy stores and that tissue oxygenation was adequate during the incubation (154). The fiber type composition of epitrochlearis muscle (6-12% Type I, 10-20% Type IIa, 70-80% Type IIb) is similar to that of total hindlimb muscle mass (5% Type I, 19% Type IIa, 76% Type IIb) (6, 154). It is also highly activated during swim exercise (based on glycogen depletion, increased glucose transport, and activation of AMPK after exercise compared to sedentary control) (5, 54, 154).

Swim exercise were used in some experiments in this thesis. Swim exercise by rats is a well characterized model for studying the effect of exercise on skeletal muscle glucose transport. Unlike treadmill exercise, swim exercise does not require familiarization that could potentially have residual effects on sedentary controls. As mentioned above, rat epitrochlearis muscle is recruited during swim exercise (154). Rat epitrochlearis muscle sampled immediately after swim exercise has increased AMPK phosphorylation and PAS-AS160, but not Akt phosphorylation (5, 144), similar to human skeletal muscle biopsy sampled immediately after aerobic exercise (102, 138). Also in rat epitrochlearis muscle, swim exercise leads to: 1) an increase in glucose transport immediately post-exercise (5, 26, 74, 173); and 2) a subsequent increase in insulin-

stimulated glucose transport several hours later (5, 23, 24, 26, 49, 61, 64, 155, 173, 174). Similar effects are also observed in human skeletal muscle after exercise (12, 68, 120, 158, 163, 164). Therefore in the context of studying skeletal muscle glucose transport, rat swim exercise is a useful model.

Some experiments in this thesis used in vitro contraction by isolated skeletal muscle. In vitro contraction provides valuable information for understanding mechanisms that are involved in skeletal muscle glucose transport during exercise. In vivo exercise activates a host of hormonal, neuronal and vascular responses in addition to the direct effect of contractile activity. Isolated skeletal muscle is useful for studying muscle contraction (17, 24, 45, 52, 89, 168) because: 1) the direct effect of skeletal muscle contraction alone can be studied; 2) duration or frequency of contraction can be precisely manipulated; 3) identical electrical stimulation is applied to muscles from different rats (muscle recruitment pattern may differ among rats during in vivo exercise); 4) the incubation environment during muscle contraction can be tightly regulated (e.g., temperature, buffer/serum, oxygenation, pH, energy substrates); 5) various inhibitors (e.g., wortmannin, Compound C, etc.) can be applied at precise concentration to isolated, contracting muscles; 6) muscles can be frozen immediately (animals must first be anesthetized before muscles are dissected after in vivo exercise); and 7) force generated by contracting muscles can be directly measured.

Gaps to be Filled by this Research

The regulation of glucose transport in skeletal muscle is a pivotal process because skeletal muscle accounts for the majority of blood glucose clearance during insulin

stimulation and exercise (40). However, the molecular mechanisms by which insulin or exercise regulate skeletal muscle glucose transport have yet to be completely mapped out. The purpose of this research was to further examine the roles that AS160 and TBC1D1 play in contraction-stimulated glucose transport.

In Study 1, the time-courses for contraction-stimulation of glucose transport and phosphorylation of AMPK, Akt, CaMKII, AS160 and TBC1D1 were determined in rat epitrochlearis muscle. Earlier studies provided some information on the time-courses for activation of each of the contraction-stimulated kinases (AMPK, Akt and CaMKII) separately (111, 123, 124, 130, 138, 146, 148), but this research was the first to measure all of these kinases in the same muscles using the same contraction protocol. The time-course for AS160 phosphorylation with in vivo exercise by humans has been reported, but they did not include assessment of all three of these kinases or glucose transport, and the only published study on contraction-stimulated TBC1D1 phosphorylation only included data at a single time-point (143). Assessing time-courses for contraction-stimulated kinases, their potential substrates (AS160 and TBC1D1) and the functional endpoint (glucose transport) provided valuable information for designing future experiments and useful insights into the relationships among these parameters.

In Study 2, the influence of inhibiting contraction-stimulated activation of AMPK (using Compound C) or PI3K and Akt (using wortmannin) on glucose transport and phosphorylation of AS160 and TBC1D1 in rat epitrochlearis muscle were determined. Although purified AMPK was able to phosphorylate AS160 and TBC1D1 in cell-free assays, there was uncertainty about whether the contraction-induced activation of AMPK in skeletal muscle was effective for phosphorylating either protein. Several lines of

evidence support the ability of contraction-induced activation of Akt for increasing AS160 phosphorylation in skeletal muscle, but no research addressed the role of Akt in contraction-stimulated phosphorylation of TBC1D1. In addition, although AMPK was widely considered as a likely participant in contraction-stimulated glucose transport, the results with genetically modified mice were ambiguous, possibly because of redundant or compensatory mechanisms in these genetic models. Therefore, it was useful to determine if acutely inhibiting AMPK is effective at reducing contraction-stimulated glucose transport. To monitor the possibility of non-specific effects of the inhibitors, it was important to confirm that there are no non-specific effects of the inhibitors on tension development or on other key contraction-stimulated kinases that have been implicated in contraction effects on glucose transport or phosphorylation of AS160 or TBC1D1.

In Study 3 and Study 4, several interventions were used to determine if the increased AS160 phosphorylation that has been reported in skeletal muscle after in vivo exercise can be uncoupled from the enhanced insulin-stimulated glucose transport after in vivo exercise or after in vitro contraction. A great deal of research indicated that the increased insulin-stimulated glucose transport after acute exercise was not attributable to enhancement of proximal insulin signaling steps (e.g., insulin binding and phosphorylation of IR, IRS phosphorylation and PI3K association, Akt serine phosphorylation), but several studies recently indicated that AS160 phosphorylation was increased several hours after exercise in rats (5) and humans (138, 148). To probe the possible functional importance of the increase in AS160 phosphorylation, this study determined if: 1) the persistent increase in AS160 phosphorylation was consistently found after exercise or contraction under conditions that have been shown to lead to

increased insulin-stimulated glucose transport (the day after exercise in rats that have not been refed; 3 hours after contraction of isolated muscles in serum), and 2) if elevated AS160 phosphorylation was not found after exercise or contraction conditions that are not characterized by increased insulin-stimulated glucose transport (after exercise when rats are refed a high carbohydrate diet; after contraction of isolated muscles in serum-free buffer). Furthermore, the effect of in vivo exercise, with and without subsequent electrically stimulated contraction, on insulin-stimulated glucose transport was determined to see if these two stimuli (in vivo exercise and in vitro contraction) would have additive effects on insulin sensitivity.

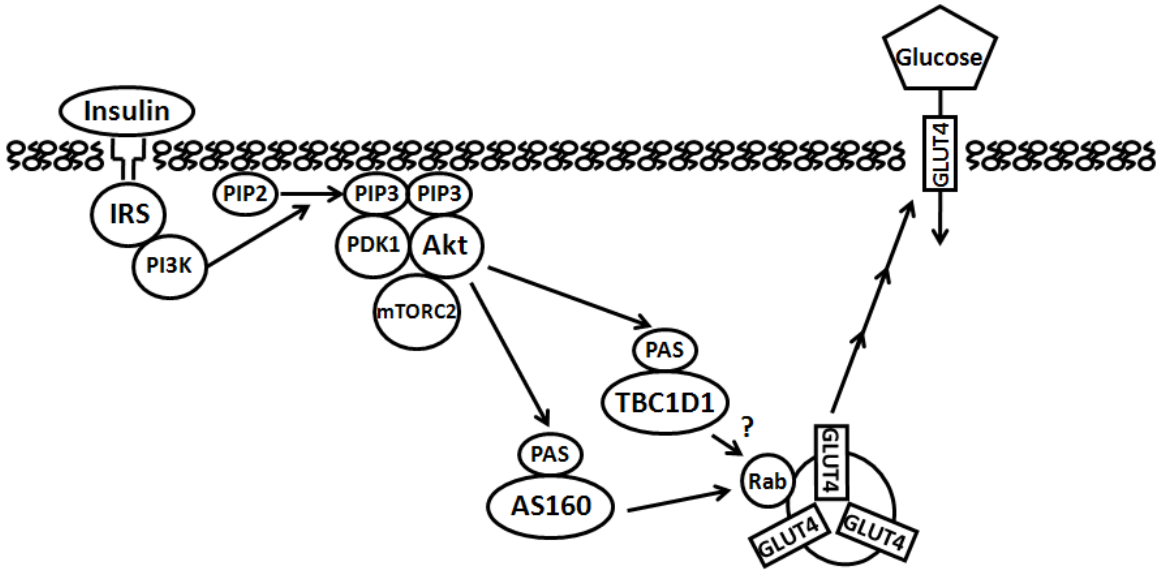
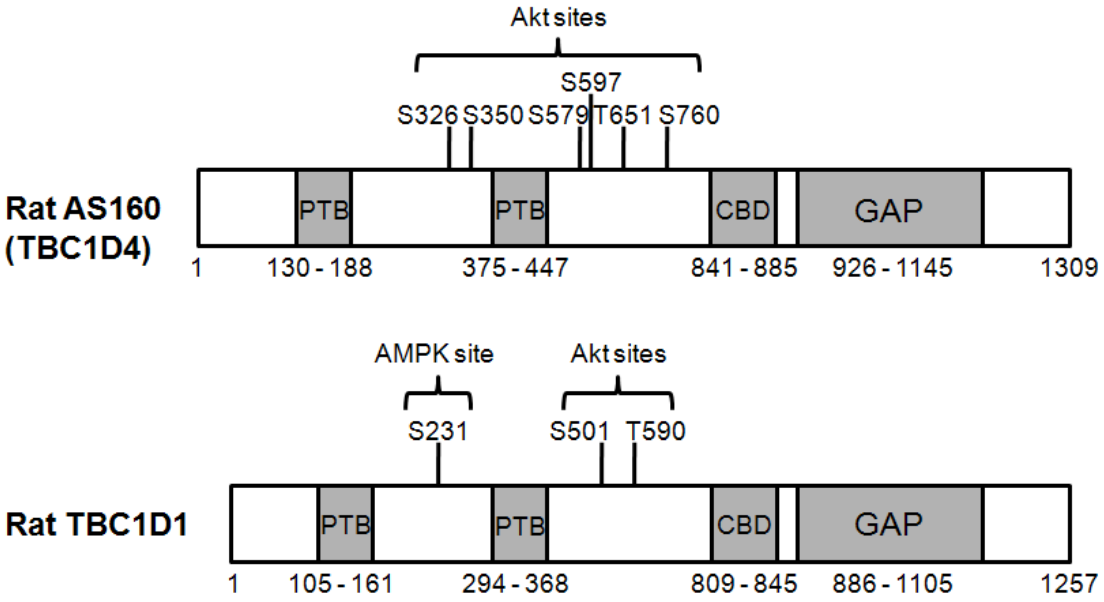


Figure 2.1
Putative Roles for AS160 and TBC1D1 for Insulin-stimulated Glucose Transport. Insulin binding to its receptor triggers a downstream signaling cascade through insulin receptor substrate (IRS), phosphatidylinsolitol 3-kinase (PI3K), phosphatidylinsolitol-(3,4,5)- trisphosphate (PIP3), phosphoinositide-dependent kinase-1 (PDK1), mammalian target of rapamycin complex-2 (mTORC2) and Akt. Akt phosphorylates AS160 and TBC1D1 on phospho-Akt substrate (PAS) motif. PAS-phosphorylation of AS160 inhibits its RabGAP activity that allows subsequent Rab-dependent GLUT4 translocation to the cell-surface membrane. The effect of insulin-stimulated PAS-phosphorylation of TBC1D1 on GLUT4 translocation is uncertain.



Not to scale. Drawn based on data from Blastp (protein-protein Basic Local Alignment Search Tool, http://blast.ncbi.nlm.nih.gov) on domains previously reported by Roach et al. (Biochem J, 2007) and Chen et al. (Biochem J, 2007).

Figure 2.2
Schematic Representations of Rat AS160 and TBC1D1. AS160 or TBC1D1 each contains a Rab GTPase-activating protein (GAP) domain that can inhibit activity of Rab-dependent vesicle trafficking. Phosphorylation of AS160 or TBC1D1 on some sites appears to result in the inactivation of RabGAP activity. There are six Akt phosphorylation sites on AS160, whereas two Akt and one AMPK phosphorylation sites exist on TBC1D1. The calmodulin binding domain (CBD) on AS160 appears to regulate contraction-stimulated, but not insulin-stimulated glucose transport. A CBD is also found in TBC1D1 but, its function has not been evaluated. AS160 or TBC1D1 also each contains two phospho-tyrosine binding domains (PTB) whose functional importance has yet to be tested. Corresponding phosphorylation sites in humans are S318, S341, S570, S588, T642, S666, S751 for AS160 and S237, S507, T596 for TBC1D1.

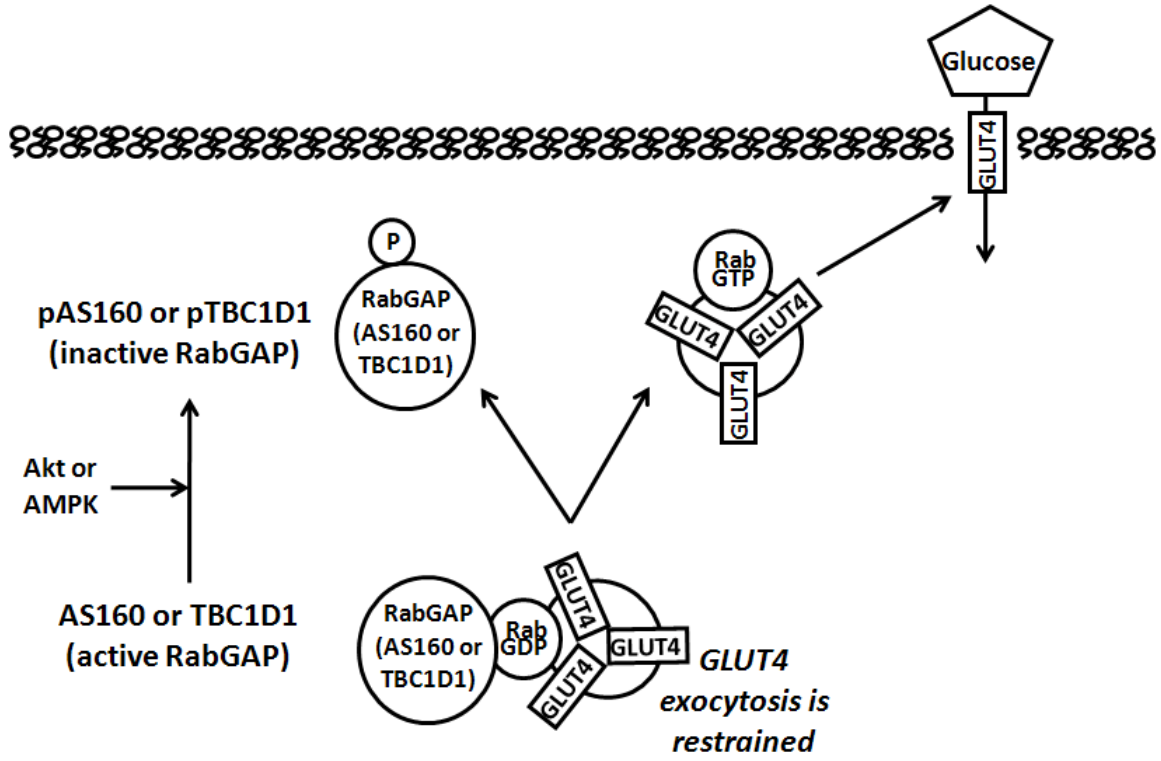


Figure 2.3
Putative Regulation of Rab proteins and GLUT4 Translocation by Phosphorylation of AS160 or TBC1D1. In the unstimulated state, unphosphorylated AS160 and/or TBC1D1 (RabGAP) are believed to be associated with GLUT4 vesicles and to inhibit Rab-dependent GLUT4 translocation through Rab-GDP formation. Upon phosphorylation by Akt and/or AMPK, RabGAP activity becomes inhibited and consequently a greater amount of Rab-GTP is present. Subsequently AS160 and/or TBC1D1 are/is dissociated from GLUT4 vesicles which are translocated in a Rab-dependent process.

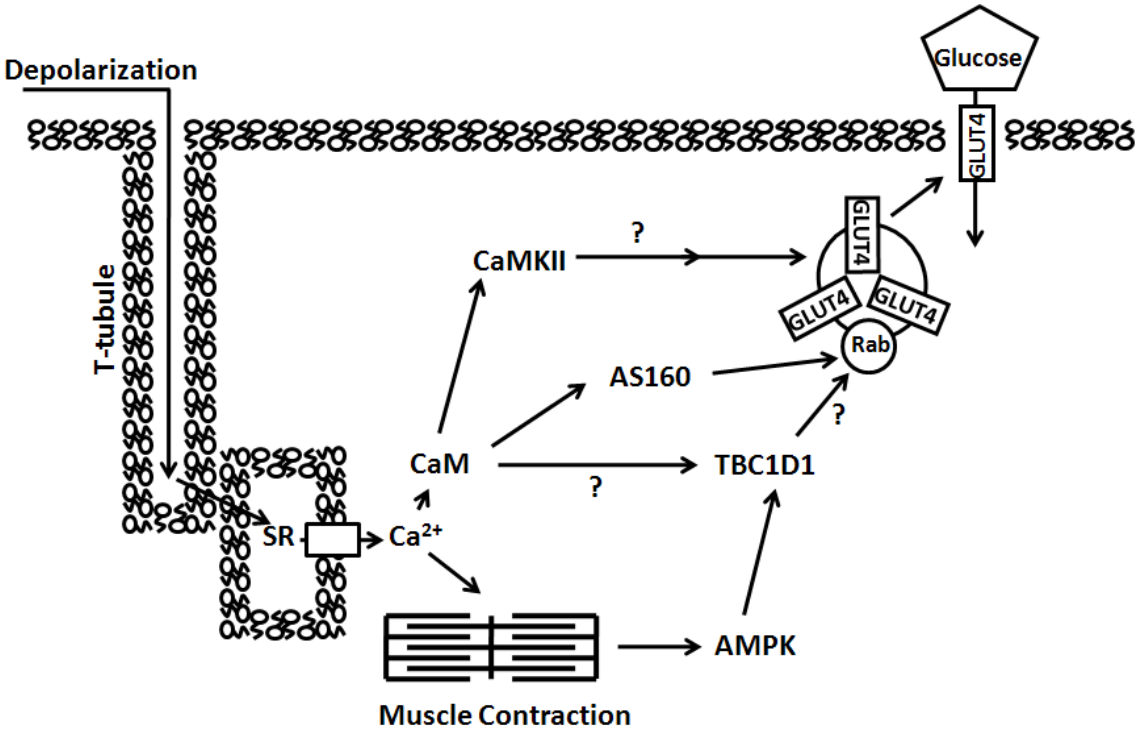


Figure 2.4
Putative Roles of AS160 and TBC1D1 for Contraction-stimulated Glucose Transport. T-tubule depolarization results in the release of calcium ions from the sarcoplasmic reticulum (SR) to the cytosol. Calcium ions bind to troponin allowing actinomyosin interaction and tension development. Muscle contraction leads to the increase in intracellular AMP concentration (secondary to activation of myosin ATPase and SR Ca^{2+} ATPase) that leads to the activation of AMPK. Activated AMPK phosphorylates TBC1D1 that results in the inactivation of RabGAP activity, promoting Rab-dependent GLUT4 translocation. Calcium ions released from the SR also bind to calmodulin (CaM). CaM can bind to AS160 during contraction and potentially regulate GLUT4 translocation through a Rab-dependent process. The function of TBC1D1's CaM binding domain is unknown. CaM can also bind to CaMKII causing autophosphorylation. Inhibitors of CaMKII have been shown to reduce contraction-stimulated glucose transport.

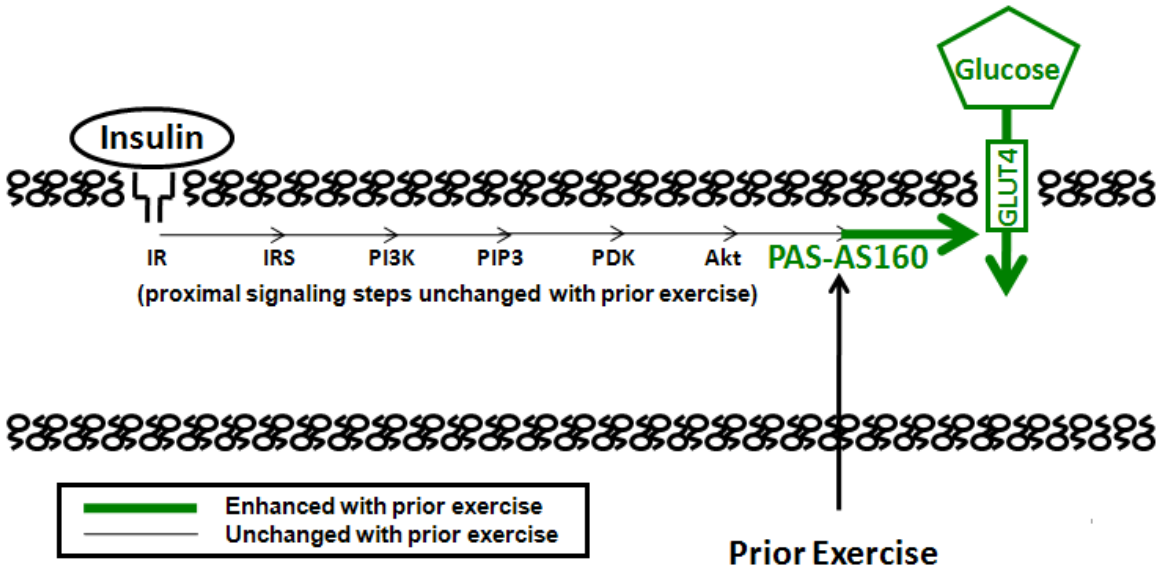


Figure 2.5
Proposed Role of PAS-AS160 in the Post-Exercise Increase in Insulin-stimulated Glucose Transport. The post-exercise increase in insulin-stimulated glucose transport occurs as a result of a post-exercise increase in insulin-stimulated GLUT4 translocation. However, proximal insulin signaling steps (IR binding, pIR, IRTK, pIRS, IRS-PI3K, pAkt) that lead to insulin-stimulated GLUT4 translocation are not enhanced by prior exercise. In contrast, PAS phosphorylation of AS160 (PAS-AS160) in exercised rats remains elevated above sedentary controls 4 hours post-exercise. It seems conceivable that the sustained increase in PAS-AS160 post-exercise plays a role in post-exercise increase in insulin-stimulated glucose transport.

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CHAPTER III

STUDY 1

Contraction-stimulated Glucose Transport in Rat Skeletal Muscle is Sustained despite Reversal of Increased PAS-phosphorylation of AS160 and TBC1D1

ABSTRACT

Akt substrate of 160kD (AS160), the most distal insulin signaling protein known to be important for insulin-stimulated glucose transport, becomes phosphorylated with skeletal muscle contraction. Akt, AMP-activated protein kinase (AMPK) and Ca^{2+}/calmodulin-dependent kinase (CaMK)-II have been implicated in regulating AS160 and/or glucose transport. Our primary aim was to assess time-courses for contraction's effects on glucose transport and phosphorylation of Akt, AMPK, CaMKII, and AS160. Isolated rat epitrochlearis muscles were studied without or with contraction (5, 10, 20, 40, 60min). Phospho-Akt substrate (PAS) antibody was used to measure AS160 PAS-phosphorylation by quantifying the ~160kD band on PAS immunoblots (PAS-160); a separate band at 150kD (PAS-150) that responded similarly to contraction was also identified. Using specific antibodies for AS160 or TBC1D1 on immunoblots, the molecular weight of PAS-160 was found to correspond with AS160 and not TBC1D1, whereas PAS-150 corresponded with TBC1D1 and not AS160. Furthermore, supernatant of sample immunodepleted with anti-AS160 had greatly reduced PAS-160, whereas

supernatant of sample immunodepleted with anti-TBC1D1 had greatly reduced PAS-150, providing further evidence that PAS-160 and PAS-150 correspond with PAS-AS160 and PAS-TBC1D1, respectively. Contraction induced transient increases in PAS-160, PAS-150, pGSK3 (an Akt substrate) and pCaMKII; glucose transport and pAMPK increases were maintained for 60min of contraction. These data suggest: 1) PAS-160 (AS160) and PAS-150 (TBC1D1) respond to contraction transiently despite sustained stimulation; 2) continual AMPK activation was insufficient for sustained increase in PAS-160 or PAS-150; and 3) sustained elevation of PAS-160 or PAS-150 was unnecessary to maintain contraction-stimulated glucose transport for up to 60min.

INTRODUCTION

Insulin or contractile activity each result in a rapid increase in glucose transport by isolated rat skeletal muscle that can be sustained for at least 60 min with continuous stimulation (11, 20). Although each stimulus induces the redistribution of GLUT4 glucose transporters from the cell's interior to its surface, multiple lines of evidence indicate that they trigger translocation by distinct mechanisms (5, 8). For example, combining maximally effective insulin and contractile activity results in an essentially additive increase in glucose transport (7, 19), and concentrations of the phosphatidylinositol 3-kinase inhibitor wortmannin that completely inhibit insulin-stimulated glucose transport do not alter contraction-stimulated glucose transport (16, 36).

Sano et al. (26) demonstrated that Akt Substrate of 160 kDa (AS160) phosphorylation is a key step linking the insulin signaling pathway with GLUT4 translocation in 3T3L1 adipocytes. Subsequently, Bruss et al. (3), using isolated rat

skeletal muscle, found rapid (half-time of ~2.5 min) and sustained increases in the phosphorylation of Akt (pAkt) and AS160 (measured using the Phospho Akt Substrate, PAS antibody, half-time ~7min), consistent with the time-course for insulin's effect on glucose transport in isolated skeletal muscle (11). Experiments using wortmannin (3), an Akt inhibitor (10), Akt knockdown by short hairpin RNA (10), or Akt2 null mice (24) provide substantial evidence for Akt being the major insulin-stimulated kinase that phosphorylates AS160.

Insulin and contractile activity appear to regulate glucose transport by distinct mechanisms, but because muscle contraction can activate Akt (25), it was not completely unexpected that contraction by isolated muscle in the absence of insulin also increased AS160 PAS phosphorylation (3). Incubation of isolated skeletal muscle with AICAR resulted in increased PAS-AS160 (3) suggesting that AMP-activated protein kinase (AMPK) might also be capable of phosphorylating AS160, which was confirmed by Treebak et al. (32) who reported that incubation of recombinant AMPK with AS160 caused an increase in PAS-AS160.

Activation of Akt is not essential for contraction-stimulated glucose transport (24, 36), whereas AMPK and Ca^{2+}/calmodulin-dependent kinase II (CaMKII) have each been implicated to be involved in contraction-stimulated glucose transport (34). Previous research has demonstrated transient, contraction-mediated activation of Akt (17, 25) and CaMKII (22, 23), but sustained AMPK activation during contraction (28, 30). However, because the time-courses for activation of these kinases were determined in separate studies using different contraction protocols, it would be valuable to assess the activation of each kinase, together with PAS-AS160, in the same muscles stimulated by the same

contraction protocol. Accordingly, the primary aim of this study was to evaluate the time-courses for contraction-stimulated effects on phosphorylation of Akt, AMPK, CaMKII and AS160 in isolated rat skeletal muscle. To better understand contraction-induced modulation of the three contraction-stimulated kinases, we also evaluated the phosphorylation of glycogen synthase kinase 3 (GSK3), acetyl CoA carboxylase (ACC) and serum response factor (SRF), substrates for Akt, AMPK and CaMKII, respectively.

We previously found that wortmannin could completely eliminate the contraction-induced increase in PAS-AS160 of isolated rat epitrochlearis muscles, suggesting that Akt may be the dominant kinase for increasing PAS-AS160 under these conditions (3). Therefore, we hypothesized that contractile activity would result in a transient increase in pAkt and PAS-AS160. We further hypothesized that the same contraction protocol would transiently activate CaMKII, but induce a sustained activation of AMPK and glucose transport.

METHODS

Materials. Reagents and apparatus for SDS-PAGE and immunoblotting including Precision Plus Protein Dual Color Standards (no. 161-0734) were from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay reagent (no. 23227) and T-PER® tissue protein extraction reagent (no. 78510) were from Pierce Biotechnology (Rockford, IL). Anti-phospho-Thr^{308}Akt (pThrAkt, no. 9275), anti-phospho-Ser^{473}Akt (pSerAkt, no. 9271), anti-phospho-Ser$^{21/9}$GSK3α/β (pGSK3, no 9331), anti-phospho-Thr^{172}AMPK (pAMPK, no. 2531), anti-phospho-Ser^{79}ACC (pACC, no. 3661), anti-phospho-Thr^{286}CaMKII (pCaMKII, no. 3361), anti-phospho-Ser^{103}SRF (pSRF, no. 4261), anti-

phospho-(Ser/Thr) Akt substrate (PAS, no. 9611), and goat anti-rabbit IgG HRP conjugate (no. 7074) were purchased from Cell Signaling Technology (Danvers, MA). PAS recognizes Akt phosphorylation motif peptide sequences (RXRXXpT/S). TBC1D1 polyclonal antibody was provided by Dr. Jianxin Xie (Cell Signaling Technology). AS160 antibody (no. 07-741) was purchased from Upstate USA (Charlottesville, VA). Preclearing Matrix F (no. 45057) and ExactaCruz™ F-HRP (no. 45043) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal (West Dura Extended Duration Substrate; Pierce, no. 34075) was used to visualize immunoblots. 3-*O*-methyl-[^{3}H]glucose ([^{3}H]3-MG) was from Sigma-Aldrich (St. Louis, MO), and [^{14}C]mannitol was from Perkin Elmer (Waltham, MA). Other reagents were 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. Male Wistar rats (~150–200 g; Harlan, Indianapolis, IN) were provided with rodent chow (Lab Diet; PMI Nutritional International, Brentwood, MO), and water ad libitum until 1700 the night before experiment and did not have access to food thereafter. On the next day, between 1000 and 1300, rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (~60 mg/kg wt). While rats were under deep anesthesia, both epitrochlearis muscles were rapidly dissected out.

Muscle treatment. Epitrochlearis muscles were incubated in glass vials containing Krebs-Henseleit buffer (KHB) + 0.1 % bovine serum albumin (BSA) + 8 mM glucose (Solution 1) and were shaken for 30 min in a water bath at 35°C with continuous gassing (95 % O_2/5 % CO_2). Muscles were then mounted in a water-jacketed glass vessel

that was warmed using a temperature-controlled bath (35°C). The distal end of the muscle was attached to a glass rod, and the proximal end was attached to a force transducer (Radnoti, Litchfield, CT) as previously described (9). The mounted muscles were incubated in KHB + BSA + glucose with continuous gassing (95% O_2/5% CO_2) and were stimulated to contract (Grass S48 Stimulator; Grass Instruments, Quincy, MA) for 5, 10, 20, 40 or 60 min (2 ms twitch, 120 twitch/min) or rested (0, 5 or 60 min). Subsequently, some muscles were rapidly blotted, trimmed of connective tissue, and freeze-clamped with aluminum tongs cooled to the temperature of liquid N_2 and then stored at –80°C until homogenization and analysis. Other muscles were transferred to vials containing KHB + 2 mM pyruvate + 36 mM mannitol (Solution 2) at 30°C for 10 min prior to being used for determination of glucose transport rate.

Measurement of glucose transport. After the 10 min incubation in Solution 2, muscles were transferred to flasks containing KHB, 0.1 % BSA with 8 mM 3-MG (including [^{3}H]3-MG 0.25 mCi/mmol), and 2 mM mannitol (including [^{14}C]mannitol 0.1 mCi/mmol). After incubation with 3-MG for 10 min, the muscles were rapidly blotted on filter paper dampened with incubation media, trimmed, freeze-clamped, and stored at –80°C until processed as described below.

Homogenization. Frozen muscles used for glucose transport and immunoblotting (PAS, pSerAkt, pThrAkt, pGSK3, pAMPK, pACC, pCaMKII, and pSRF) were homogenized in 1 ml ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % IGEPAL, 2 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerophosphate, 1 mM phenylmethanesulphonylfluoride, and 1 µg/ml leupeptin) using glass-on-glass tubes (Kontes, Vineland, NJ). Homogenates were

subsequently rotated at 4°C for 1 h before being centrifuged (12,000 *g* for 10 min at 4°C). Aliquots of the supernatant from muscles used for the 3-MG analysis were pipetted into vials for scintillation counting, and 3-MG accumulation was determined as previously described (4). A portion of the supernatant was used to determine protein concentration by the bicinchoninic acid assay (27), and the remainder was stored at –80°C until it was further analyzed.

Immunoprecipitation. Frozen muscles to be immunoprecipitated with anti-AS160 or anti-TBC1D1 were homogenized in T-PER supplemented homogenization buffer (2 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerophosphate, 1 mM phenylmethanesulphonylfluoride, and 1 µg/ml leupeptin in T-PER®). Homogenized muscle (300-500 µg protein) was precleared in preclearing matrix F for 30 min and the resulting supernatant was immunoprecipitated with 1.5–2 µg of anti-AS160 or anti-TBC1D1 at 4°C using ExactaCruz™ F-HRP. After gentle rotation overnight, the immunoprecipitation mix was centrifuged at 4,000*g*, and the immunodepleted supernatant was aspirated and subsequently used for immunoblotting. After washing (four times with 500 µl phosphate-buffered saline), the protein bound to the beads was eluted with 2x SDS loading buffer and boiled before loading on a polyacrylamide gel.

Immunoblotting. Immunoprecipitates, immunodepleted supernatants or homogenized muscle lysates in the SDS loading buffer were separated and electrophoretically transferred to nitrocellulose. Samples 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 % nonfat dry milk in TBST for 1 h at room temperature,

washed 3 x 5 min at room temperature, and treated 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 secondary antibody, goat anti-rabbit IgG HRP conjugate (1:20,000 in TBST + 5 % milk), for 1 h at room temperature, washed again 3 x 5 min with TBST, and developed with SuperSignal reagent. Protein bands were quantitated by densitometry (Alpha Innotech, San Leandro, CA). The mean values for resting samples on each blot were normalized to equal 1.0, and then all samples on the blot were expressed relative to the normalized resting value.

Statistical analysis. Statistical analyses were done using Sigma Stat version 2.0 (San Rafael, CA). Data are expressed as means ± SE. $P \leq 0.05$ was considered statistically significant. One-way ANOVA was used to determine significant differences with contraction, and the source of significant variance was detected using the Dunnett post hoc test (versus resting control). When data failed the Levene Median test for equal variance, the data were transformed (base 10 logarithm) prior to performing ANOVA. A Pearson product moment correlation was used to assess the relationship between variables. For correlations determined between two signaling proteins, each correlated pair of signaling measurements was from the same muscle. Correlations determined with tension measurements also used signaling or glucose transport values from the same muscle. For correlations determined between glucose transport and a signaling value, the data were from the contralateral muscles from the same rat.

RESULTS

Rested Muscles. There were no significant effects of incubation time (0, 5 or 60 min) for protein phosphorylation or glucose transport in the rested muscles. Therefore, values for resting muscles were pooled for statistical analyses and are represented as 0 min time point in the figures (Fig. 3.2, 3.4 and 3.5).

Tension Development. Peak tension was 117.2 ± 8.7 g·g wt muscle^{-1}. There was a progressive decline in tension development reaching ~50% of the peak value at ~15 min and 19.1 ± 4.0 % of peak tension at 60 min of contraction (Fig. 3.1). The peak tension (represented at 0 min) was significantly greater than tension development at 5, 10, 20, 40 and 60 min ($P<0.05$).

Contraction-activated kinases and phosphorylated substrates. There was a transient trend for level of pSerAkt and pThrAkt to increase above resting at 5 min of contraction (Fig. 3.2A). The level of pGSK3, an Akt substrate, was significantly increased above resting at 10 min (half-time of ~5 min) and returned to baseline by 60 min (Fig. 3.2B). There was a significant contraction-induced increase in both pAMPK (10, 20, 40 and 60 min, half-time of ~7.5 min, Fig. 3.2C) and its substrate pACC (10, 20, 40 and 60 min, half-time of ~4 min, Fig. 3.2D). Contraction caused a transient increase in pCaMKII (20 min, half-time of ~4 min, Fig. 3.2E) and its substrate pSRF (20 min, half-time of ~7 min, Fig. 3.2F), and each returned to baseline by 60 min.

PAS-160 and PAS-150. When lysates prepared from rat epitrochlearis muscles were immunoblotted using anti-AS160, the AS160 band migrated above the 150 kD molecular weight marker (Fig 3.3A, lane 1). In samples that were immunoprecipitated using anti-AS160 before immunoblotting with anti-PAS, the contraction-responsive PAS-band was also visualized above the 150 kD molecular weight marker (PAS-AS160, Fig.

3.3A, lane 2). For samples that were immunoblotted using anti-PAS without prior immunoprecipitation, a contraction-responsive PAS band was visible at a site corresponding to the location of the AS160 (Fig. 3.3A lane 1) and PAS-AS160 (Fig 3.3A, lane 2) bands, and it was designated as PAS-160 (Fig 3.3A, lane 3). There was strong PAS-immunoreactivity above the 150 kD marker in the anti-AS160 immunoprecipitate of contraction-stimulated muscle (PAS-AS160; Fig. 3.3B, lane 2), but the PAS-160 signal was barely detectable in the adjacent lane which had been loaded with supernatant of the anti-AS160 immunodepleted sample (Fig. 3.3B lane 1). These results suggest that PAS-AS160 accounts for the PAS-160 band of samples that had not been immunoprecipitated. PAS-160 was significantly greater than the resting value at 10 min of contraction (half-time of ~7 min), reached its peak value at 20 min and returned to baseline by 60 min of contraction (Fig. 3.4A).

On the same immunoblots, we also observed a contraction-responsive PAS band at ~150 kD (Fig 3.3A, lane 3 and 3.3C lane 3, designated PAS-150). Recently, a paralog protein of AS160 called TBC1D1 has been identified as a novel substrate of Akt that may also be involved in insulin-stimulated GLUT4 translocation in adipocytes (21). TBC1D1 has been reported to have a slightly lower apparent molecular weight compared to AS160 and therefore it seemed possible that PAS-150 was TBC1D1 (29). Supporting this idea, when lysates were immunoblotted using anti-TBC1D1, the TBC1D1 band migrated at ~150 kD (Fig. 3.3C, lane 1). In samples that were immunoprecipitated using anti-TBC1D1 before immunoblotting with anti-PAS, the contraction-responsive PAS band corresponded to PAS-150 (PAS-TBC1D1, Fig. 3.3C, lane 2), providing supporting evidence that PAS-150 may include PAS-TBC1D1. Furthermore, the supernatant of anti-

TBC1D1 immunoprecipitated sample had only a barely detectable PAS-150 signal (Fig. 3.3D lane 1) compared to the strong PAS-signal in the anti-TBC1D1 immunoprecipitate (PAS-TBC1D1; Fig 3.3D lane 2). These findings provide additional evidence that PAS-TBC1D1 accounts for the PAS-150 band of samples that had not been immunoprecipitated. PAS-150 peaked at 20 min of contraction (half-time of ~10 min), at which time it was significantly greater than resting values. PAS-150 returned to baseline at 60 min (Fig. 3.4B).

Glucose Transport. Contraction resulted in a rapid (half-time of ~8 min) and significant increase in glucose transport (10, 20, 40 and 60 min). The peak value occurred at 20 min and plateaued thereafter (Fig. 3.5).

Correlations. Pearson correlation analyses revealed that (Table 3.1), PAS-160 was significantly ($P<0.01$) correlated only with pGSK3 ($R=0.629$), pCaMKII ($R=0.724$) and PAS-150 ($R=0.776$). 3-MG transport was significantly ($P<0.01$) correlated only with pAMPK ($R=0.350$). Tension was not significantly correlated with 3MG transport or any of the signaling proteins studied.

DISCUSSION

The primary aim of this study was to assess the time-courses for contractile activity on tension development, phosphorylation of three contraction-stimulated kinases (Akt, AMPK, CaMKII), PAS-160 and glucose transport. In an earlier study, we found that a brief and discontinuous tetanic stimulation protocol (10 sec tetanus duration, 2 tetani/min for 5 min) which induced a large activation of Akt can also increase AS160 phosphorylation (3). For the current study, we instead used a twitch contraction protocol

(2 ms twitch, 120 twitch/min) because: 1) discontinuous stimulation with 20 sec recovery periods between tetani would complicate the interpretation of a time-course analysis; 2) the time-course for glucose transport by rat epitrochlearis had previously been characterized using a similar twitch contraction protocol (20); and 3) the twitch vs. tetanic protocol resulted in a slower fatigue rate. This approach revealed some useful new insights regarding contraction effects on skeletal muscle glucose transport, including: 1) identification of a contraction-responsive phosphorylated protein band (PAS-150) that appears to correspond with PAS-TBC1D1 and which migrated at a slightly lower apparent molecular weight (MW) than AS160; 2) demonstration that a sustained increase in neither PAS-150 nor PAS-160 (which appears to correspond with PAS-AS160) was essential for maintenance of elevated glucose transport with 40 or 60 min of stimulation; and 3) recognition that the values for PAS-150 and PAS-160 from muscles after contraction correlated with each other and with pGSK3 (an Akt substrate) or pCaMKII, but not with pAMPK or glucose transport.

The commercially available PAS antibody was designed to identify unknown Akt substrates by reacting with proteins that are phosphorylated on Akt consensus motifs (RXRXXpS/T). PAS immunoblots prepared using homogenates of rat epitrochlearis muscles had multiple PAS-reactive bands at various MW including two that were consistently increased for contraction vs. resting samples at an apparent MW of ~150 kD and above 150 kD. The band visible above the 150 kD marker presumably includes AS160 because immunoreactivity against the AS160 antibody corresponds to the same location on immunoblots. Immunoprecipitation using anti-AS160 followed by immunoblotting with PAS also identified an AS160-associated PAS band at the same

location (Fig. 3.3A). Furthermore, the supernatant of sample immunodepleted with the AS160 antibody had a greatly reduced PAS-160 signal (Fig. 3.3B). The PAS-150 band responded similarly to PAS-160 in response to the contraction protocol, peaking at 20 min and reversing to resting values by 60 min of contraction. During the preparation of this manuscript, Chavez et al. (6) and Taylor et al. (29) reported that TBC1D1 protein is much more abundant in skeletal muscle than in adipose tissue and that TBC1D1 becomes phosphorylated in response to AICAR (an AMPK activator) or contractile activity. The location on immunoblots for reactivity against the TBC1D1 antibody corresponded to the location of PAS-150 band suggesting that it includes TBC1D1 (Fig, 3.3C). Supporting this idea, the supernatant of sample immunodepleted using TBC1D1 antibody had a greatly reduced PAS-150 signal (Fig. 3.3D). AS160 was apparently not part of the PAS-150 band based on the slower migration of the band visualized using the AS160 antibody and the apparent lack of reduced PAS-150 signal in the AS160 immunodepleted supernatant. Furthermore, the lack of immunoreactivity against the TBC1D1 antibody on immunoblots at the location of PAS-160 and the apparent lack of reduced PAS-160 signal in the TBC1D1 immunodepleted supernatant suggests that TBC1D1 was not part of that protein band.

The reversal of the contraction-stimulated increase in PAS-160 at 40 and 60 min despite continued electrical stimulation is in contrast to the sustained increase in PAS-160 that was previously found with 60 min of in vitro insulin-stimulation of rat epitrochlearis muscle (3). It is conceivable that the reversal of the increase in PAS-160 was related, at least in part, to muscle fatigue, although the time-courses were different for the decrements in contraction-stimulation of PAS-160 and tension development. These

results with in vitro contraction were also in contrast to the published results for skeletal muscle which have indicated that: 1) in muscle biopsies taken during in vivo exercise by humans, PAS-160 is increased at 60 and 90 min, but not at 1, 10 or 30 min (31); and 2) after in vivo exercise, increase in PAS-160 is maintained for at least 2.5 to 4 hr after cessation of exercise in rats (1) or humans (13, 28). Even if reversal of PAS-160 is not typical during in vivo exercise, in vitro contraction data may reveal useful clues regarding the regulatory processes which modulate phosphorylation of AS160 and/or TBC1D1.

To begin evaluating the mechanisms that regulate AS160 phosphorylation during contraction, we compared the timecourses for contraction effects on several key kinases concomitant with PAS-160 in the same muscles. As hypothesized, contraction resulted in transient enhancement of pGSK3 and pCaMKII together with a sustained increase in pAMPK. The patterns of contraction effects on pGSK3, pCaMKII and pAMPK were consistent with previous reports (17, 22, 23, 25, 28, 30). Although it is possible that pAMPK was involved in the initial increase in PAS-160, the reversal of the increase in PAS-160 at 40 and 60 min of stimulation indicates that increased pAMPK was not sufficient for a sustained increase in PAS-160. The significant correlation between pGSK3 (an Akt substrate) and PAS-160 is consistent with the idea that Akt is the primary in vitro contraction-stimulated AS160 kinase in rat epitrochlearis, at least as recognized with the PAS antibody. This result is also in agreement with our demonstration that the phosphatidylinositol 3-phosphate inhibitor wortmannin causes full inhibition of contraction-stimulation of pAkt, PAS-AS160 (3) and PAS-160 (Arias and Cartee, unpublished data) in rat epitrochlearis. Wortmannin does not inhibit the activity of purified AMPK (2) or AMPK phosphorylation in contraction-stimulated cardiomyocytes

(35). Nonetheless, kinases other than Akt may be relevant and phosphorylation may also be occurring on sites not recognized by the PAS antibody. Furthermore, protein phosphorylation reflects the action of both kinases and phosphatases, and currently little is known about the role of phosphatases in the effects of contraction on AS160 phosphorylation. A novel result was the significant correlation between pCaMKII and PAS-160 as well as PAS-150. Both rat AS160 and rat TBC1D1 proteins contain several motifs that are potential sites of CaMKII phosphorylation (scansite.mit.edu). Of these, AS160 Ser^{597} and TBC1D1 Thr^{590} are Akt phospho-motifs (RXRXXpS/T), and the remaining CaMKII phospho-sites have arginine at the -3 position (XXRXXpS/T). Therefore, it is conceivable that CaMKII phosphorylates AS160 and TBC1D1 on PAS sites. It is also possible that CaMKII phosphorylates AS160 and/or TBC1D1 on non-PAS sites, and thereby affects contraction-stimulated increases in PAS-AS160, PAS-TBC1D1 and/or glucose transport. The relatively high correlation between PAS-160 and PAS-150 suggests that these proteins may share mechanisms that regulate the phosphorylation on PAS sites (kinases and/or phosphatases).

The only statistically significant correlation that was found between a signaling protein and glucose transport was the modest relationship between pAMPK and glucose transport. This association supports a great deal of previous evidence that AMPK is involved in contraction-stimulated glucose transport (5, 12, 18). In contrast, a persistent increase in pGSK3 or pCaMKII was not required for a sustained increase in glucose transport. The pGSK3 data are not surprising because many studies have indicated that Akt is not essential for contraction-stimulated glucose transport. However, some studies using inhibitors have suggested that CaMKII may play a role in a portion of contraction-

mediated glucose transport (33, 34). These earlier studies used relatively brief periods of contraction, and their findings are consistent with the current data with regard to an activation of pCaMKII during the initial minutes of contraction. Different processes may be required for initiation compared to the maintenance of elevated glucose transport.

Compelling evidence indicates that a contraction-induced increase in PAS-160 is not essential for increased muscle glucose transport, including: 1) the complete inhibition of contraction-stimulated PAS-AS160 in isolated rat epitrochlearis muscle (3) by concentrations of wortmannin which have no effect on contraction-stimulated glucose transport in isolated muscle (16, 36), and 2) the failure for in vitro contraction to elevate PAS-160 in muscles from α2 AMPK null mice (32) even though contraction-stimulated glucose uptake is not attenuated in the null compared to wild-type control mice (14). Furthermore, PAS-AS160 can remain elevated above basal levels for several hours after the cessation of in vivo exercise by rats (1) despite reversal of exercise-stimulated increase in insulin-independent glucose transport, indicating that elevated PAS-AS160 alone is not sufficient to increase glucose transport. A new finding that is consistent with these earlier studies was that sustained contraction resulted in temporal uncoupling of PAS-160 from contraction-stimulated glucose transport. Importantly, the current data demonstrate that sustained increase in PAS-150 (apparently TBC1D1) was also not required for maintained increase in contraction-stimulated glucose transport at 40 or 60 min.

The PAS antibody appears to have differential immunoreactivity with AS160 phosphorylated on some Akt phosphomotifs relative to others (26), and PAS immunoreactivity would presumably not be a sensitive indicator of increased

phosphorylation of AS160 or TBC1D1 on phosphomotifs for other kinases. Furthermore, there is evidence that AS160 may modulate contraction-stimulated glucose transport by a mechanism related to its calcium-calmodulin binding domain (15), so the current findings do not preclude roles for AS160 in regulating contraction-stimulated glucose transport. It remains to be determined if TBC1D1 plays a role in contraction-stimulated glucose transport.

In conclusion, the current data using an in vitro twitch contraction protocol with rat epitrochlearis muscle demonstrate that: 1) PAS-160 (apparently AS160) and PAS-150 (apparently TBC1D1) both transiently respond to a sustained stimulation protocol; 2) continual activation of AMPK was not sufficient for sustained increase in PAS-160 or PAS-150; 3) temporal relationships suggest that Akt and possibly CaMKII may be involved in the contraction-stimulated increase in PAS-160 and/or PAS-150; and 4) sustained elevation of PAS-160 or PAS-150 is not necessary for contraction-stimulated glucose transport. Other approaches will be essential to clarify the mechanisms whereby contraction regulates AS160 and/or TBC1D1 function and to reveal the specific roles of these Rab GAP proteins in the initiation, maintenance and reversal of contraction-stimulated glucose transport.

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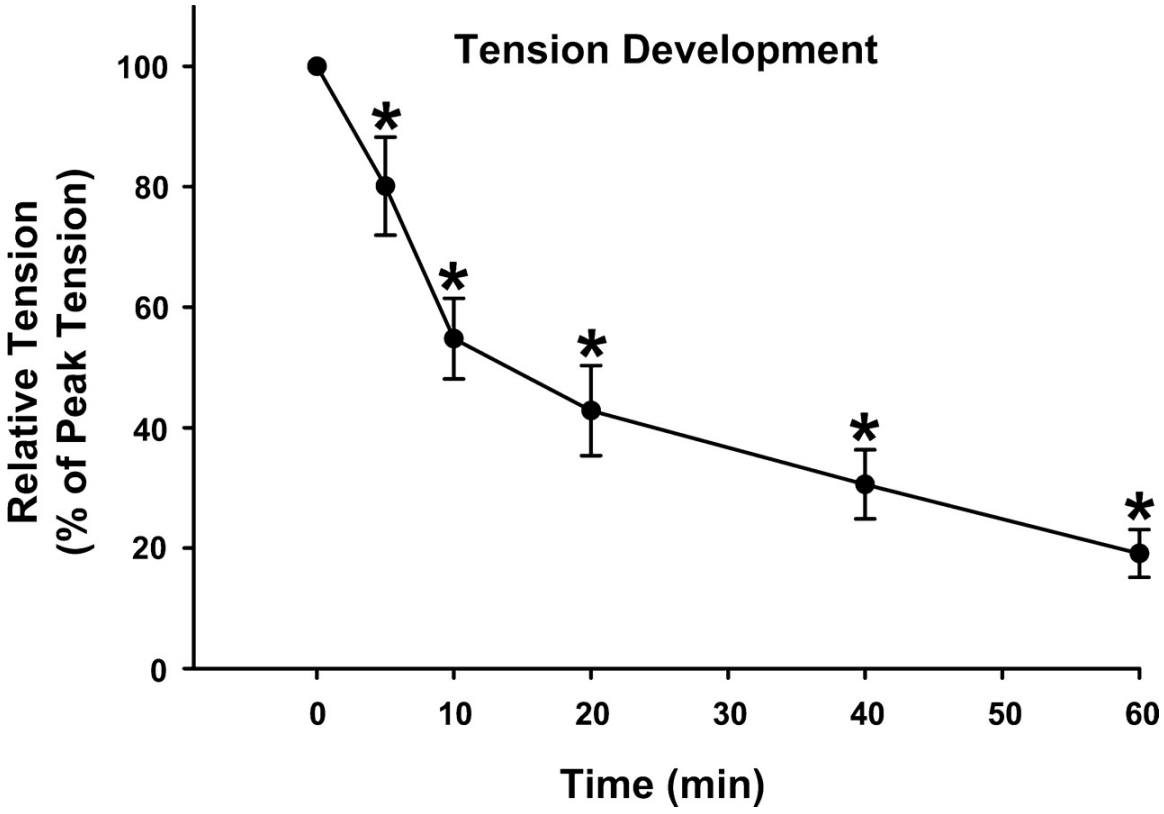

Figure 3.1
Time course for tension development in isolated rat epitrochlearis muscles that were contracted for 60 min. Data are means ± S.E., n = 15 per group. Post-hoc analysis: *P<0.05 vs. peak tension (at 0 min).

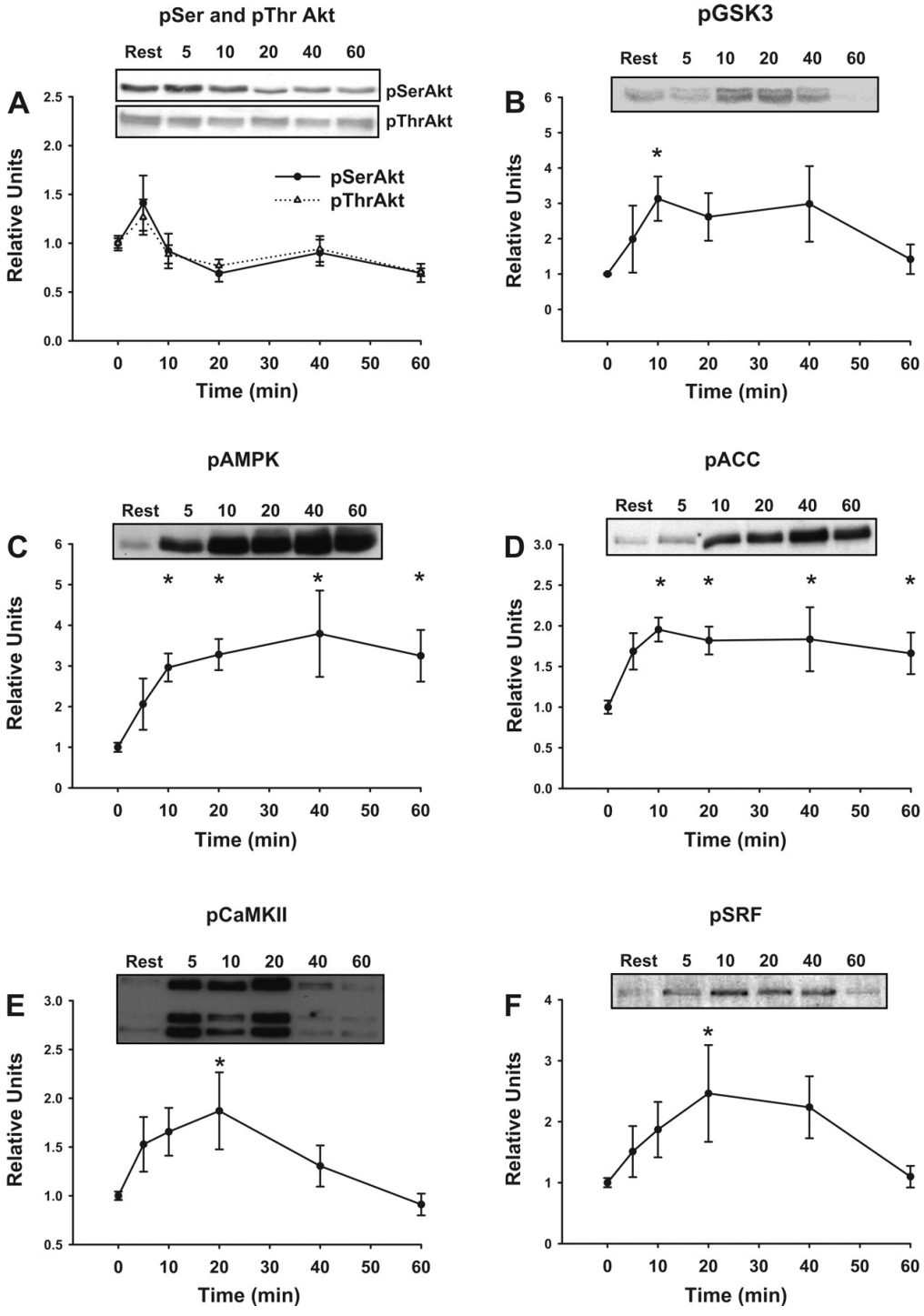

Figure 3.2
Time course for contraction-stimulated phosphorylation of Akt^{Ser473} and Akt^{Thr308}, GSK3$^{Ser21/9}$, AMPK^{Thr172}, ACC^{Ser79}, CaMKII^{Thr286} and SRF^{Ser103} in isolated rat epitrochlearis muscles. The values at 0 min are from rested muscles. Representative blots are shown. Data are means ± S.E., n = 7-12 per group. Post-hoc analysis: *P<0.05 vs. basal.

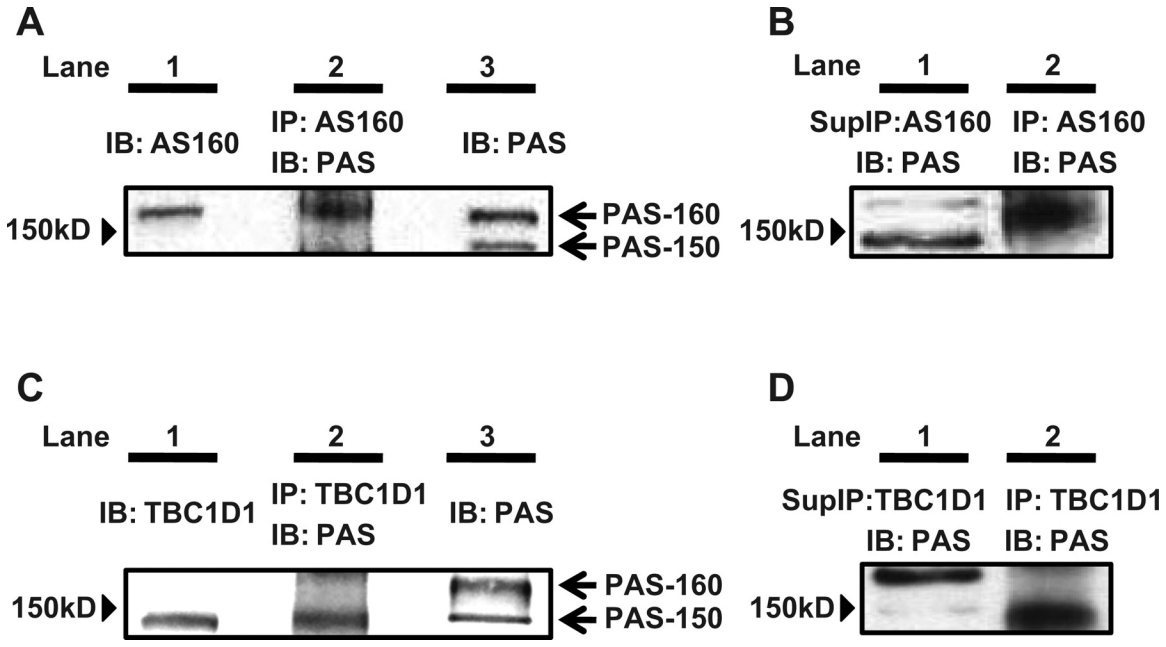

Figure 3.3
PAS-160 and PAS-150. Lysates from isolated rat epitrochlearis muscle that were stimulated to contract for 20 min were subjected to immunoblotting (with or without prior immunoprecipitation). The samples in each Panel (A, B, C or D) were run on the same gel, transferred to the same blot and then the blots were cut into strips that were separately incubated in the different primary antibodies as indicated. (A) The band in the anti-AS160 immunoblot migrated above 150 kD (Fig. 3.3A lane 1). The band for the sample undergoing anti-AS160 immunoprecipitation prior to anti-PAS immunoblot was also visualized above 150 kD (PAS-AS160, Fig. 3.3A lane 2). The anti-PAS immunoblot of muscle lysates without prior immunoprecipitation had a PAS band visible at a site corresponding to the location of the AS160 (lane 1) and PAS-AS160 (lane 2) bands and was designated as PAS-160 (Fig. 3.3A lane 3). (B) AS160-immunodepleted supernatant had a greatly reduced PAS-160 signal (Fig. 3.3B lane 1), indicating that PAS-AS160 (Fig. 3.3B lane 2) accounts for the PAS-160 band of samples that had not been immunoprecipitated. (C) On the anti-PAS immunoblot, we also observed a PAS band at ~150 kD (Fig. 3.3A lane 3 and 3.3C lane 3, designated PAS-150). Anti-TBC1D1 immunoblot (Fig. 3.3C lane 1) and anti-TBC1D1 immunoprecipitation before anti-PAS immunoblot (PAS-TBC1D1, Fig. 3.3C lane 2) each had a band at ~150 kD, corresponding to the location of PAS-150. (D) TBC-1D1-immunodepleted supernatant had a greatly reduced PAS-150 signal (Fig. 3.3D lane 1), indicating that PAS-TBC1D1 (Fig. 3.3D lane 2) accounts for the PAS-150 band of samples that had not been immunoprecipitated.

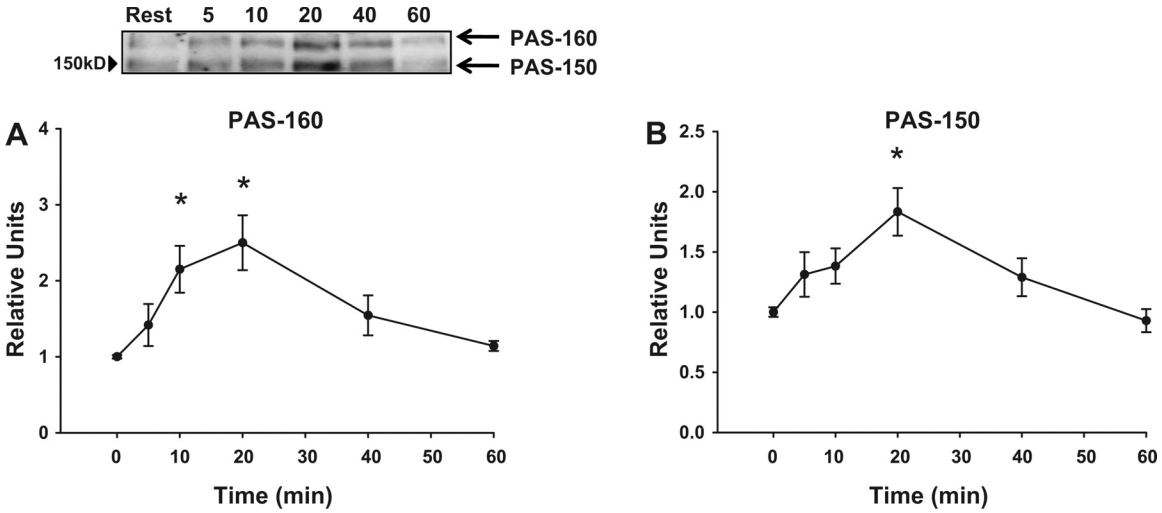

Figure 3.4
Time course for contraction-stimulated PAS-160 and PAS-150 in isolated rat epitrochlearis muscles. The values at 0 min are from rested muscles. Representative blots are shown. Data are means ± S.E., n = 7-16 per group. Post-hoc analysis: *P<0.05 vs. basal.

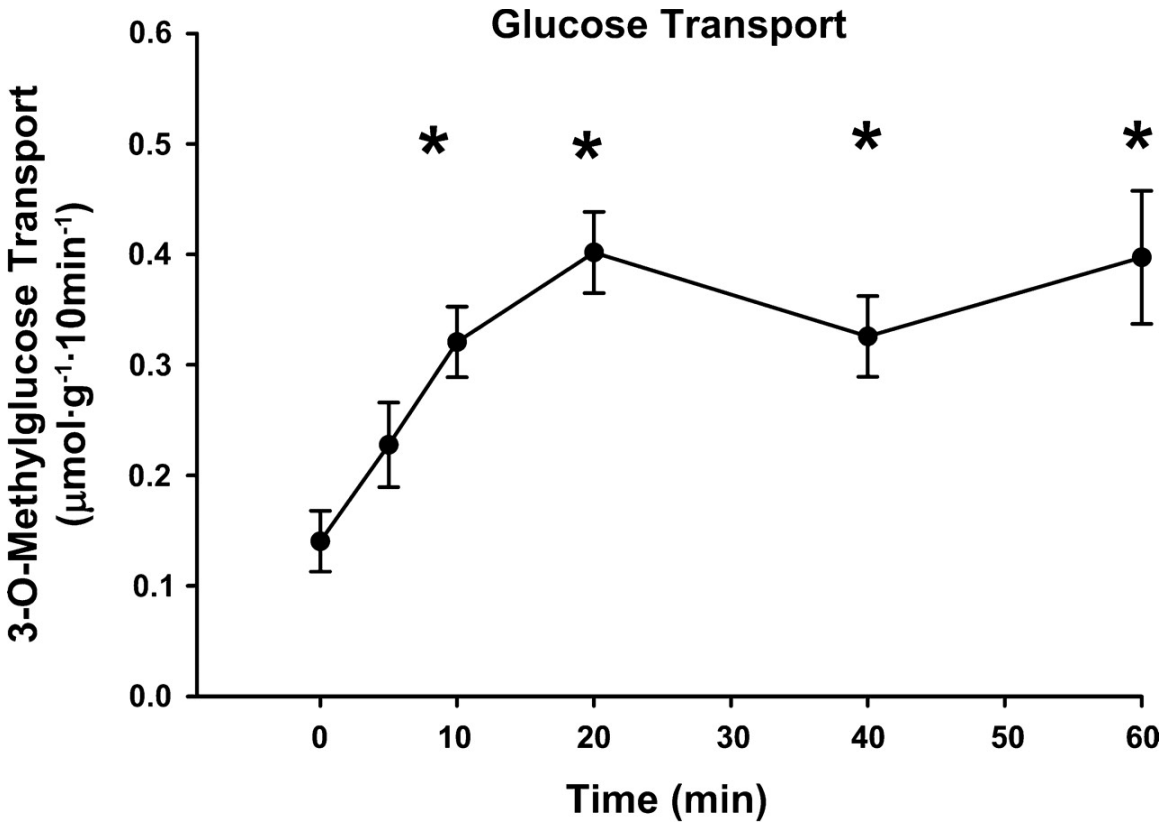

Figure 3.5
Time course for contraction-stimulated glucose transport in isolated rat epitrochlearis muscles. The values at 0 min are from rested muscles. The rate of glucose transport was measured using [^{3}H]3-O-methylglucose (3-MG). Data are means ± S.E., n = 12-18 per group. Post-hoc analysis: *P<0.05 vs. basal.

	pGSK3	pAMPK	pCaMKII	PAS-160	PAS-150
PAS-160	0.629*	–0.053	0.724*		0.776*
PAS-150	0.660*	0.044	0.666*	0.776*	
Glucose transport	–0.033	0.350*	0.257	0.118	0.078

Table 3.1
R values for Pearson product-moment correlation analyses. The *R* values for Pearson product-moment correlations are indicated in the matrix. Each point on correlations between two signaling proteins is from the same muscle. Each point used for the correlation between a signaling protein and glucose transport is from paired muscles. n = 41–51. pGSK3, phosphorylation of glycogen synthase kinase 3; pAMPK, phosphorylation of AMP-activated protein kinase; pCaMKII, phosphorylation of Ca^{2+}/calmodulin-dependent kinase II; PAS, phospho-Akt substrate. * $P < 0.01$.

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CHAPTER IV

STUDY 2

Inhibition of Contraction-stimulated AMPK Inhibits Contraction-stimulated Increases in PAS-TBC1D1 and Glucose Transport without Altering PAS-AS160 in Rat Skeletal Muscle

ABSTRACT

Phosphorylation of two members of the TBC1 domain family of proteins, Akt substrate of 160kD (AS160, also known as TBC1D4) and TBC1D1, has been implicated in the regulation of glucose transport in skeletal muscle. Insulin-stimulated phosphorylation (measured using the phospho-Akt substrate, PAS, antibody) of AS160 and TBC1D1 appears to occur in an Akt-dependent manner, but the kinases responsible for contraction-stimulated PAS-AS160 and PAS-TBC1D1 remain unclear. AMP-activated protein kinase (AMPK) and Akt, both activated by contraction, can each phosphorylate AS160 and TBC1D1 in cell-free assays. To evaluate the roles of AMPK and Akt on insulin- or contraction-stimulated PAS-AS160, PAS-TBC1D1 and glucose transport, rat epitrochlearis were incubated ± Compound C (inhibitor of AMPK) or wortmannin (inhibitor of phosphatidylinositol 3-kinase, PI3K, which is upstream of Akt) prior to and during insulin-stimulation or contraction. Insulin-stimulated glucose transport and phosphorylation of both AS160 and TBC1D1 were completely inhibited by

wortmannin. Wortmannin eliminated contraction stimulation of pGSK3 (Akt substrate) and PAS-AS160, but did not significantly alter pAMPK, pACC (AMPK substrate), PAS-TBC1D1 or glucose transport in contraction-stimulated muscle. Compound C completely inhibited contraction-stimulated pACC and PAS-TBC1D1 and partially blocked glucose transport, but did not significantly alter pAkt, pGSK3 or PAS-AS160. These data suggest that: 1) insulin stimulates glucose transport and phosphorylation of AS160 and TBC1D1 in a PI3K/Akt-dependent manner; 2) contraction stimulates PAS-AS160 (but not PAS-TBC1D1 or glucose transport) in a PI3K/Akt-dependent manner; and 3) contraction-stimulates PAS-TBC1D1 and glucose transport (but not PAS-AS160) in an AMPK-dependent manner.

INTRODUCTION

Insulin and contractile activity, the two most important physiologic stimuli that increase glucose transport in skeletal muscle, can each induce the translocation of GLUT4 glucose transporters from the cell's interior to its surface membranes (9, 11). However, they regulate glucose transport via distinct signaling pathways (5). Insulin-stimulated glucose transport requires phosphatidylinositol 3-kinase (PI3K) activation, which leads to Akt activation without stimulating AMP-activated protein kinase (AMPK) (5, 8, 20, 32). A great deal of evidence suggests that contraction stimulates glucose transport by a mechanism independent of PI3K/Akt (19, 31, 39, 48) and attributable to the effects of multiple inputs, with AMPK- and calcium-mediated processes being major factors (35, 47).

In 3T3-L1 adipocytes, insulin stimulates phosphorylation of Akt substrate of 160kD (AS160; also called TBC1D4) in an Akt-dependent manner on sites identifiable by the phospho-Akt substrate (PAS) antibody (25, 41). AS160 includes a Rab GTPase-activating protein domain (RabGAP) that inhibits Rab proteins which are involved in regulating vesicular traffic (34). The insulin-mediated increase in PAS-phosphorylation of AS160 (PAS-AS160) appears to inhibit RabGAP activity, thereby allowing GLUT4 to be recruited to surface membranes and elevate glucose transport (13, 34, 41, 49). In skeletal muscle, insulin or contraction results in elevated PAS-AS160 (3, 15), and AS160 phosphorylation appears to regulate glucose transport (29).

Recently, TBC1D1, a RabGAP protein paralog to AS160, was also shown to become PAS-phosphorylated (PAS-TBC1D1) in response to insulin in an Akt-dependent manner (38). However, whereas AS160 knockdown in 3T3-L1 adipocytes resulted in elevated basal cell-surface GLUT4 (1, 13), TBC1D1 knockdown had no effect on basal cell-surface GLUT4 in 3T3-L1 cells (6). TBC1D1 protein is only ~5% as abundant as AS160 protein in 3T3-L1 adipocytes, which may explain why TBC1D1 does not appear to play a major role in regulating glucose transport in these cells (6). TBC1D1 protein abundance is much greater in skeletal muscle versus adipose tissue (43), and silencing TBC1D1 in L6 myotubes resulted in increased basal cell-surface GLUT4 (22), supporting the idea that TBC1D1 inhibits GLUT4 translocation in the basal state. However, in contrast to the results for L6 cells with AS160 knockdown (which did not alter the insulin-stimulated net increase in cell-surface GLUT4), silencing TBC1D1 in L6 cells resulted in greater insulin-induced GLUT4 translocation versus control cells (22). In other words, TBC1D1 knockdown allowed insulin to induce a greater amount of GLUT4

translocation than in cells that express TBC1D1. These findings suggest that at least a portion of the inhibitory effects of TBC1D1 on GLUT4 may not be restrained by insulin. However, they do not eliminate the possibility that TBC1D1 can regulate an insulin-independent increase in glucose transport (e.g., with contraction). PAS-TBC1D1 is elevated in response to contraction in rodent skeletal muscle (15, 43). Therefore, it seems possible that PAS-TBC1D1 may play a role in mediating contraction-stimulated glucose transport.

Experiments using purified Akt or AMPK demonstrated that each kinase can phosphorylate both AS160 and TBC1D1 in cell-free assays (7, 17). Considerable evidence indicates that the insulin-stimulated increase in PAS-AS160 is Akt-dependent in skeletal muscle (3, 28), and increased AS160 phosphorylation appears to be important for the full effect of insulin on glucose transport (29). However, the specific kinases responsible for contraction-stimulated PAS-AS160 need to be clarified because: 1) wortmannin can completely inhibit the contraction-stimulated increase in PAS-AS160 in rat skeletal muscle suggesting that Akt is responsible for the increased PAS-phosphorylation of AS160 during contraction (3); but 2) muscles from mice with genetically disrupted AMPK versus wild-type littermates had reduced contraction-stimulated increase in immunoreactivity towards PAS antibody at ~160 kD (PAS-160) (28, 45).

The primary aim of this study was to elucidate the contributions of Akt and AMPK on increases in PAS-AS160 and PAS-TBC1D1 in skeletal muscle stimulated by insulin or contraction. The PI3K inhibitor wortmannin was used to prevent Akt activation (without altering AMPK activation), and Compound C, a potent AMPK

inhibitor (50), was used to prevent AMPK activation (without altering Akt activation). A secondary aim was to determine if inhibition of insulin- or contraction-stimulated increases in PAS-AS160 or PAS-TBC1D1 was accompanied by attenuated insulin- or contraction-stimulated glucose transport. We hypothesized that in isolated rat epitrochlearis muscle: 1) Akt-dependent mechanisms are essential for the insulin-stimulated increases in glucose transport and phosphorylation of AS160 and TBC1D1; 2) Akt-dependent (but not AMPK-dependent) mechanisms are essential for contraction-stimulated increases in PAS-AS160, but not glucose transport; and 3) AMPK-dependent (but not Akt-dependent) mechanisms are essential for contraction-stimulated increases in PAS-TBC1D1 (but not PAS-AS160) and glucose transport.

METHODS

Materials. Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). Wortmannin was purchased from Sigma-Aldrich (St. Louis, MO). Compound C was from EMD Chemicals, Inc. (San Diego, CA). Reagents for SDS-PAGE and immunoblotting including Precision Plus Protein Dual Color Standards were from Bio-Rad (Hercules, CA). Bicinchoninic acid protein assay reagent, T-PER tissue protein extraction reagent and West Dura Extended Duration Substrate were from Pierce Biotechnology (Rockford, IL). Anti-phospho-Thr^{308}Akt (pAkt), anti-phospho-Ser$^{21/9}$Glycogen Synthase Kinase 3α/β (pGSK3), anti-GSK3α, anti-phospho-Thr^{172}AMPK (pAMPK), anti-AMPK, anti-phospho-Ser^{79}Acetyl CoA Carboxylase (pACC), anti-ACC, anti-phospho-Thr^{286}Ca^{2+}/calmodulin-dependent kinase II (pCaMKII), anti-CaMKII, anti-phospho-(Ser/Thr) Akt substrate [PAS which was designed to

recognize Akt phosphorylation motif peptide sequences (RXRXXpT/S)], and goat anti-rabbit IgG HRP conjugate were from Cell Signaling Technology (Danvers, MA). TBC1D1 polyclonal antibody was provided by Dr. Jianxin Xie (Cell Signaling Technology). AS160 antibody was from Millipore (Billerica, MA). Anti-Akt1/2/3, Preclearing Matrix F and ExactaCruz F-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA). 3-*O*-methyl-[^{3}H]glucose ([^{3}H]3-MG) was from Sigma-Aldrich, and [^{14}C]mannitol was from Perkin Elmer (Waltham, MA). Other reagents were 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 (~150–200 g; Harlan, Indianapolis, IN) were provided with rodent chow (Lab Diet; PMI Nutritional International, Brentwood, MO) and water ad libitum until 1700 the night before the experiment when their food was removed. The next day, between 1000 and 1300, rats were anesthetized (intraperitoneal injection of sodium pentobarbital; 60 mg/kg wt). While rats were deeply anesthetized, both epitrochlearis muscles were rapidly extracted.

Muscle Treatment. Isolated epitrochlearis muscles were pre-incubated in Krebs-Henseleit buffer (KHB) + 0.1% bovine serum albumin (BSA) + 8 mM glucose (Solution 1) in a water bath at 35°C with continuous gassing (95% O_2/5% CO_2). During this step, one muscle per rat was incubated in Solution 1 with either 500 nM wortmannin (30 min) or 40 μM Compound C (60 min); stock solutions of each inhibitor were dissolved in vehicle, dimethyl sulfoxide (DMSO), and the contralateral muscle was incubated in Solution 1 containing vehicle (wortmannin, 0.05% DMSO; Compound C, 0.4% DMSO). Inhibitors or vehicle remained at the same concentration throughout subsequent

incubations. Some muscles were transferred to vials containing identical media for 20 min (Basal). Other muscles were attached to a glass rod and force transducer (Radnoti, Litchfield, CT) as previously described (12). Mounted muscles were incubated in Solution 1 with gassing (95% O_2/5% CO_2) and stimulated to contract as previously described (Grass S48 Stimulator; Grass Instruments, Quincy, MA; 20 min, 2 ms pulse, 120 pulses/min, 25V) (15). Immediately afterward, muscles were either freeze-clamped or transferred to vials containing KHB + 2 mM pyruvate + 36 mM mannitol (Solution 2; 30°C, 10 min) prior to 3-MG transport measurement.

In separate experiments, muscles pre-incubated with inhibitors or vehicle were transferred to vials with Solution 1 supplemented with either insulin (2000 μU/mL, 20 min) or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, 2 mM, 40 min). Muscles were then either freeze-clamped or transferred to vials with Solution 2 for 10 min prior to 3-MG transport measurement.

Measurement of 3-MG transport. After 10 min incubation in Solution 2, muscles were transferred to flasks containing KHB, 0.1% BSA with 8 mM 3-MG (including [^{3}H]3-MG at a final specific activity of 0.25 mCi/mmol), and 2 mM mannitol (including [^{14}C]mannitol at a final specific activity of 6.25 μCi/mmol) (4). After 10 min, muscles were rapidly blotted, trimmed, freeze-clamped, and stored (–80°C) until processed.

Homogenization. Muscles used for glucose transport and immunoblotting were homogenized in 1 ml ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% IGEPAL CA-639, 2 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 2.5 mM sodium pyrophosphate, 20 mM ß-glycerophosphate, 2 mM phenylmethanesulphonylfluoride, and 1 μg/ml leupeptin) using glass-on-glass tubes

(Kontes, Vineland, NJ). Homogenates were rotated (4°C, 1 h) before being centrifuged (12,000 *g*, 10 min, 4°C). Aliquots of supernatant used for 3-MG transport measurement were pipetted into vials for scintillation counting, and 3-MG transport was determined (4). A portion of supernatant was used to determine protein concentration by the manufacturer's instructions (Pierce Biotechnology Catalog no. 23227). Remaining supernatant was stored at –80°C until further analyzed.

Immunoprecipitation. Muscles to be immunoprecipitated by anti-PAS or anti-TBC1D1 were homogenized in T-PER-supplemented homogenization buffer (2 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerophosphate, 1 mM phenylmethanesulphonylfluoride, and 1 µg/ml leupeptin in T-PER). Homogenate (300-500 µg protein) was mixed with preclearing matrix F (30 min) and the supernatant was immunoprecipitated with 1.5–2 µg of anti-PAS, anti-AS160 or anti-TBC1D1 (4°C) using ExactaCruz F-HRP. After gentle rotation overnight, the immunoprecipitation mix was centrifuged (4,000*g*), and supernatant was aspirated. After washing (four times with 500 µl phosphate-buffered saline), protein bound to beads was eluted with 2x Laemmli sample buffer, boiled and loaded on a polyacrylamide gel.

Immunoblotting. Immunoprecipitates or lysate, boiled with SDS loading buffer, were separated and electrophoretically transferred to nitrocellulose. Samples were 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 20), blocked with 5% nonfat dry milk in TBST (1 h, room temperature), washed 3x5 min (room temperature), and treated with primary antibodies (1:1,000 in TBST + 5% BSA) overnight (4°C). Blots were washed 3x5 min with TBST, incubated with the secondary antibody, goat anti-rabbit IgG HRP conjugate (1:20,000 in

TBST + 5% milk; 1 h, room temperature), washed again 3x5 min with TBST, and developed with West Dura Extended Duration Substrate reagent. Protein bands were quantitated by densitometry (Alpha Innotech, San Leandro, CA). Mean values for basal muscles incubated without inhibitors on each blot were normalized to equal 1.0. All values were expressed relative to the normalized basal value.

Statistical analysis. Statistical analyses used Sigma Stat version 2.0 (San Rafael, CA). Data are expressed as mean ±SE. $P \leq 0.05$ was considered statistically significant. One-way ANOVA and the Student-Newman-Keuls post-hoc test were used. When data failed the Levene Median test for equal variance, the Kruskal-Wallis nonparametric ANOVA on ranks was used with Dunn's post-hoc test.

RESULTS

Tension Development. Neither wortmannin nor Compound C affected peak force or total force (data not shown).

Total Protein Abundance. For all comparisons of immunoblot band intensities, equal amounts of total protein or of immunoprecipitate derived from equal amounts of total protein were loaded in each lane. Abundance of total proteins (Akt, GSK3, AMPK, ACC, CaMKII, AS160 and TBC1D1) was unaltered by insulin, contraction, wortmannin and/or Compound C (Fig. 4.1).

AS160 and TBC1D1 Phosphorylation. As previously shown (15), the anti-PAS immunoblot of muscle samples (basal, insulin or contraction) without prior immunoprecipitation contained multiple PAS bands, including; 1) an anti-PAS band migrating at a location above the 150 kD molecular marker (PAS-160) that corresponded

to the location of the anti-AS160 band from an AS160 immunoblot; and 2) an anti-PAS band migrating at a location similar to the 150 kD molecular marker (PAS-150) and corresponding with the location of the anti-TBC1D1 band from a TBC1D1 immunoblot (data not shown). When samples were immunoprecipitated with anti-PAS and immunoblotted with anti-AS160, the location of the band (PAS-AS160) corresponded to the location of the PAS-160 band. When samples were immunoprecipitated with anti-TBC1D1 and immunoblotted with anti-PAS, the location of the band (PAS-TBC1D1) corresponded to the location of the PAS-150 band. Throughout the manuscript, when samples were directly immunoblotted with anti-PAS, the bands are referred as PAS-160 or PAS-150, whereas when samples are immunoprecipitated prior to immunoblotting, the respective bands are referred as PAS-AS160 or PAS-TBC1D1.

Wortmannin. Incubation of skeletal muscle with wortmannin for 30 min completely inhibited the insulin-stimulated increases in pAkt and pGSK3 (Fig. 4.2A-B, $P<0.001$). Insulin (with or without wortmannin) did not alter pAMPK, pACC or pCaMKII (data not shown). Insulin-stimulated increases in PAS-160 (Fig. 4.2C), PAS-150 (Fig. 4.2D) and glucose transport (Fig. 4.2E) were completely inhibited by wortmannin ($P<0.001$).

Contraction resulted in a significant increase in glucose transport, pGSK3, pAMPK, pACC, pCaMKII (Fig. 4.3 and 4.4, $P<0.05$), PAS-160 and PAS-150 (data not shown). PAS-AS160 and PAS-TBC1D1 were also significantly ($P<0.05$) elevated after contraction compared to basal muscles (Fig. 4.3F-G). Consistent with previous results using this contraction protocol (15), there was a non-significant trend for a small contraction-stimulated increase in pAkt (Fig. 4.3A and 4.4A). There were no contraction

effects on pAkt and pGSK3 in wortmannin-treated muscles (Fig. 4.3A-B, $P<0.05$). Wortmannin did not affect contraction-stimulated pAMPK, pACC or pCaMKII (Fig. 4.3C-E). Increases in PAS-AS160 (Fig. 4.3F, $P<0.01$) and PAS-160 (data not shown) in contraction-stimulated muscles were eliminated with wortmannin treatment. In contrast, increases in PAS-TBC1D1 (Fig. 4.3G) and PAS-150 (data not shown) in contraction-stimulated muscles were not significantly affected by wortmannin treatment. As expected, glucose transport in contraction-stimulated muscles was unaltered by wortmannin (Fig. 4.3H).

Compound C. Compound C caused complete inhibition of the contraction-stimulated increase in pACC (Fig. 4.4D, $P<0.001$) without affecting pAkt, pGSK3 and pCaMKII (Fig. 4.4A, B, E). Compound C did not significantly alter the increased PAS-AS160 (Fig. 4.4F) or PAS-160 (data not shown) in contraction-stimulated muscles. In contrast, Compound C significantly reduced the increases in PAS-TBC1D1 (Fig. 4.4G, $P<0.05$) and PAS-150 (data not shown) in contraction-stimulated muscles. The increase in glucose transport in contraction-stimulated muscles was partially reduced by Compound C (Fig. 4.4H, $P<0.001$). Insulin-stimulated glucose transport was unaffected by Compound C (Fig. 4.5A), and the increase in glucose transport in AICAR-stimulated muscles was completely eliminated by Compound C (Fig. 4.5B, $P<0.01$).

DISCUSSION

This study provides new information about the regulation and function of AS160 and TBC1D1, two related RabGAP proteins expressed by skeletal muscle, each of which has been implicated to modulate glucose transport. The results demonstrate that it is

possible to separate contraction's ability to increase AS160 phosphorylation from TBC1D1 phosphorylation, as identified using the PAS-antibody, and reveal novel insights regarding their respective roles in the activation of glucose transport. The data suggest that in isolated rat epitrochlearis muscle: 1) PI3K-dependent (and presumably Akt-dependent) mechanisms are essential for the insulin-stimulated increases in glucose transport and phosphorylation of AS160 and TBC1D1; 2) PI3K/Akt-dependent (but not AMPK-dependent) mechanisms are essential for the contraction-stimulated increase in PAS-AS160 (but not PAS-TBC1D1 or glucose transport); and 3) AMPK-dependent (but not PI3K/Akt-dependent) mechanisms are essential for the contraction-stimulated increases in PAS-TBC1D1 (but not PAS-AS160) and glucose transport. The findings support the idea that elevated PAS-TBC1D1, via an AMPK-dependent mechanism, may participate in contraction-mediated glucose transport.

Regarding insulin-stimulation, the data are consistent with earlier research in 3T3-L1 adipocytes (18, 25, 49), human primary myocytes (2) and rodent skeletal muscle (3, 28) which indicated that the insulin-stimulation of PAS-AS160 is Akt-dependent. Our results confirm that insulin can induce increased PAS-TBC1D1 in skeletal muscle (43). Wortmannin has been shown to reduce PAS-TBC1D1 in insulin-stimulated HEK-293 cells (38), but the current data are apparently the first demonstration in an authentic insulin-target tissue that wortmannin-induced inhibition of Akt eliminates the insulin-stimulated increase in PAS-150, which corresponds to PAS-TBC1D1.

Contraction for 20 min caused an increase in phosphorylation of GSK3, an Akt substrate, despite only a trend for increased Akt phosphorylation. These results are consistent with previous observations indicating that contractile activity can transiently

activate Akt, achieving peak activity and phosphorylation at ~2 to 5 min with reversal at ~15-20 min despite continued stimulation (15, 40). In rat epitrochlearis, contraction-stimulated PAS-AS160 was completely wortmannin-inhibitable, suggesting that contraction-stimulated AS160 PAS-phosphorylation is PI3K/Akt-dependent, as previously reported (3). The current results confirm recent studies that found contraction causes an increase in skeletal muscle PAS-TBC1D1 (15, 43). In striking contrast to AS160, wortmannin did not attenuate the contraction-stimulated increase in PAS-TBC1D1 suggesting that contraction's effect on PAS-TBC1D1 was not PI3K/Akt-dependent. Furthermore, the AMPK inhibitor Compound C completely suppressed contraction-stimulated PAS-TBC1D1 without inhibiting contraction's effect on PAS-AS160. Notably, wortmannin did not alter phosphorylation of AMPK or ACC (an AMPK substrate), and Compound C did not alter phosphorylation of Akt or GSK3 (an Akt substrate). These experiments reveal fundamental differences in mechanisms whereby contraction regulates phosphorylation of two closely related RabGAP proteins.

The data provide new evidence that increased PAS-TBC1D1 in skeletal muscle with contraction is AMPK-dependent. Supporting this interpretation, incubating skeletal muscle with AICAR induced an increase in PAS-TBC1D1 and incubation of recombinant AMPK with immunoprecipitated TBC1D1 caused increased PAS-TBC1D1 (43). Earlier studies which found that muscle from mice with genetically disrupted AMPK had reduced contraction-stimulated phosphorylation at ~160 kD did not perform immunoprecipitation of samples prior to immunoblotting with the PAS antibody (28, 45). Perhaps in these studies the PAS-immunoreactivity included both TBC1D1 and AS160,

with TBC1D1 accounting for at least a portion of the apparently AMPK-dependent phosphorylation.

A secondary aim was to determine if inhibition of insulin- or contraction-stimulated increases in PAS-AS160 or PAS-TBC1D1 were accompanied by attenuation of glucose transport. A great deal of evidence supports the idea that the insulin-stimulated increase in AS160 phosphorylation, via a PI3K/Akt-dependent mechanism, is important for increased GLUT4 translocation and glucose transport (3, 13, 18, 25, 34, 41, 49). The current data are consistent with this role of AS160. Few studies have evaluated TBC1D1's possible role in insulin-stimulated glucose transport. In 3T3-L1 adipocytes, knockdown of TBC1D1 did not alter basal or insulin-stimulated GLUT4 in surface membranes (6). However, these results are not necessarily predictive of skeletal muscle which, compared to 3T3-L1 cells, expresses TBC1D1 at much greater levels (6). In L6 myotubes, knockdown of TBC1D1 resulted in elevated basal surface GLUT4 and ~1.5-fold elevation in insulin-stimulated GLUT4 translocation (22). The authors' interpretation was that TBC1D1 can modulate GLUT4 traffic, but insulin may not regulate TBC1D1's RabGAP activity. The current data demonstrate that insulin regulates TBC1D1 phosphorylation in skeletal muscle via a PI3K/Akt-dependent mechanism, but the functional consequences of TBC1D1 phosphorylation on glucose transport remain uncertain.

Because contraction-stimulated glucose transport was unaltered by wortmannin despite elimination of the increase in PAS-AS160, it is evident that increased PAS-AS160 is not essential for contraction-mediated glucose transport. The lack of a wortmannin effect on contraction-stimulated increases in PAS-TBC1D1 and glucose

transport, together with Compound C inducing full inhibition of contraction's effect on PAS-TBC1D1 and partial inhibition of glucose transport, is consistent with the possibility that PAS-TBC1D1 participates in the contraction-stimulated increase in glucose transport. However, the current results do not establish causality, and although Compound C did not alter tension development, pCaMKII, pAkt, pGSK3 or PAS-AS160, these results do not prove that Compound C's effects are exclusively attributable to inhibiting AMPK.

Wortmannin can completely inhibit contraction's activation of Akt in skeletal muscle without reducing contraction-stimulated glucose transport (31, 36, 48). Another PI3K inhibitor (LY294002) also inhibits contraction-activated Akt (40). These results with two distinct PI3K-inhibitors suggest that PI3K is upstream of contraction-stimulated Akt. However, muscle contraction does not increase class Ia PI3K activity associated with insulin receptor substrate proteins (19) or class II PI3K activity (42). Sakamoto et al. (40) proposed that contraction may activate class Ib PI3K, but this possibility remains to be experimentally confirmed. Regardless, wortmannin can clearly eliminate contraction-stimulated increases in pAkt and PAS-AS160 in rat epitrochlearis muscle, which begs the question: how can activation of Akt and PAS-AS160 be important for insulin-stimulated glucose transport, but not contraction-stimulated glucose transport? A similar paradox is that either insulin or contraction can individually lead to increased glucose transport in skeletal muscle by increasing GLUT4 translocation, but the combined stimulation of muscle with maximally effective insulin and contraction can increase glucose transport (10, 16) and cell-surface GLUT4 (16) more than either stimulus alone. There is evidence suggesting that insulin and contraction recruit different intracellular pools of GLUT4 vesicles (9, 11). Little is known about subcellular localization of AS160 or TBC1D1, but

it has been reported that in 3T3-L1 adipocytes, AS160 appears to be associated with GLUT4 vesicles under basal conditions, and insulin-treatment can cause an increase in cytosolic AS160 (30). A speculative scenario is that insulin-recruitable GLUT4 vesicles may associate with AS160, whereas contraction-associated GLUT4 vesicles may associate with TBC1D1. In addition, AS160 and TBC1D1 function are likely also regulated by other mechanisms, including: 1) phosphorylation on sites undetectable with anti-PAS; 2) Ca^{2+}-calmodulin interaction with each protein's calmodulin binding domain; 3) binding to 14-3-3 proteins; and 4) changes in subcellular localization (7, 17, 24, 27, 30, 37, 38). Evaluation of these and alternative mechanisms will be necessary to fully understand the regulation and roles of AS160 and TBC1D1.

We also determined if acute inhibition of the contraction-stimulated activation of AMPK would result in attenuated glucose transport. AMPK was originally recognized as a potential participant in contraction-stimulated glucose transport by Winder and co-workers. They found that exercise or contraction can activate AMPK (21, 46), and AICAR, which also leads to AMPK activation, can stimulate glucose uptake (33). Many studies have supported a role for AMPK in contraction-stimulated glucose transport (20, 26, 35, 47), but research using mice with genetic modification of AMPK suggests that AMPK may not be essential for contraction-stimulated glucose transport (14, 23). With genetic modifications, there may be compensatory responses. Compound C, which offers a useful alternative approach for rapid inhibition of AMPK, resulted in reduced contraction-stimulated glucose transport, providing novel evidence that AMPK may be important for this contraction effect.

The partial reduction in contraction-stimulated glucose transport by Compound C supports earlier studies which indicated that AMPK-independent mechanisms can account for a portion of the contraction-stimulated glucose transport (35, 47). Multiple lines of evidence suggest that increased cytosolic calcium contributes to contraction-stimulated glucose transport (27, 47). For example, calmodulin binding to AS160 has been implicated in contraction-stimulated glucose transport (27). TBC1D1 also contains a calmodulin binding domain (38). Unaltered contraction-stimulated increases in pCaMKII and tension in Compound C-treated muscles suggest that cytosolic Ca^{2+} was not reduced, which may account, at least in part, for the residual effect of contraction on glucose transport. AICAR can activate AMPK without altering cytosolic Ca^{2+} concentration in isolated rat epitrochlearis (44). In this context, it is notable that, in contrast to the partial inhibition of contraction-stimulated glucose transport, Compound C completely inhibited AICAR-stimulated glucose transport. Furthermore, Compound C had no effect on insulin-stimulated glucose transport, indicating that Compound C's ability to reduce glucose transport activated by contraction or AICAR is not because of a non-specific effect on glucose transport, regardless of the stimulation pathway.

The contraction-stimulated increases in PAS-TBC1D1 and glucose transport with 20 min of contractile activity in the current study are similar to earlier results using the same contraction protocol (15). The previous study, which included assessment for up to 60 min of contraction, found that the glucose transport rate achieved with 20 min of contraction was sustained with contraction lasting 60 min even though contraction-stimulated PAS-TBC1D1 had returned to basal levels at 60 min (15). These results suggest PAS-TBC1D1 may trigger the initial increase in contraction-stimulated glucose

transport. It remains unclear if the initial increase in PAS-TBC1D1 is sufficient to cause a sustained increase in contraction-stimulated glucose transport or if another mechanism is required for a sustained increase.

In conclusion, Figure 4.6 represents our working model for increasing PAS-AS160 and PAS-TBC1D1 with insulin or contraction, and for the roles that insulin- or contraction-stimulated PAS-AS160 and PAS-TBC1D1 may play in the regulation of glucose transport: 1) insulin stimulates PAS-phosphorylation of AS160 and TBC1D1 in an Akt-dependent manner, and PAS-AS160 appears to be important for the regulation of insulin-stimulated glucose transport with the functional role of insulin-stimulated PAS-TBC1D1 currently uncertain; 2) contraction stimulates PAS-phosphorylation of AS160, but not TBC1D1, in an Akt-dependent manner, and the contraction-stimulated PAS-AS160 does not contribute to contraction-stimulated glucose transport; and 3) contraction stimulates PAS-phosphorylation of TBC1D1 in an AMPK-dependent manner, consistent with the idea that contraction-stimulated PAS-TBC1D1 may regulate contraction-stimulated glucose transport. Additional research with specific manipulation of TBC1D1 expression and/or activation will be needed to further elucidate TBC1D1's role in glucose transport of skeletal muscle.

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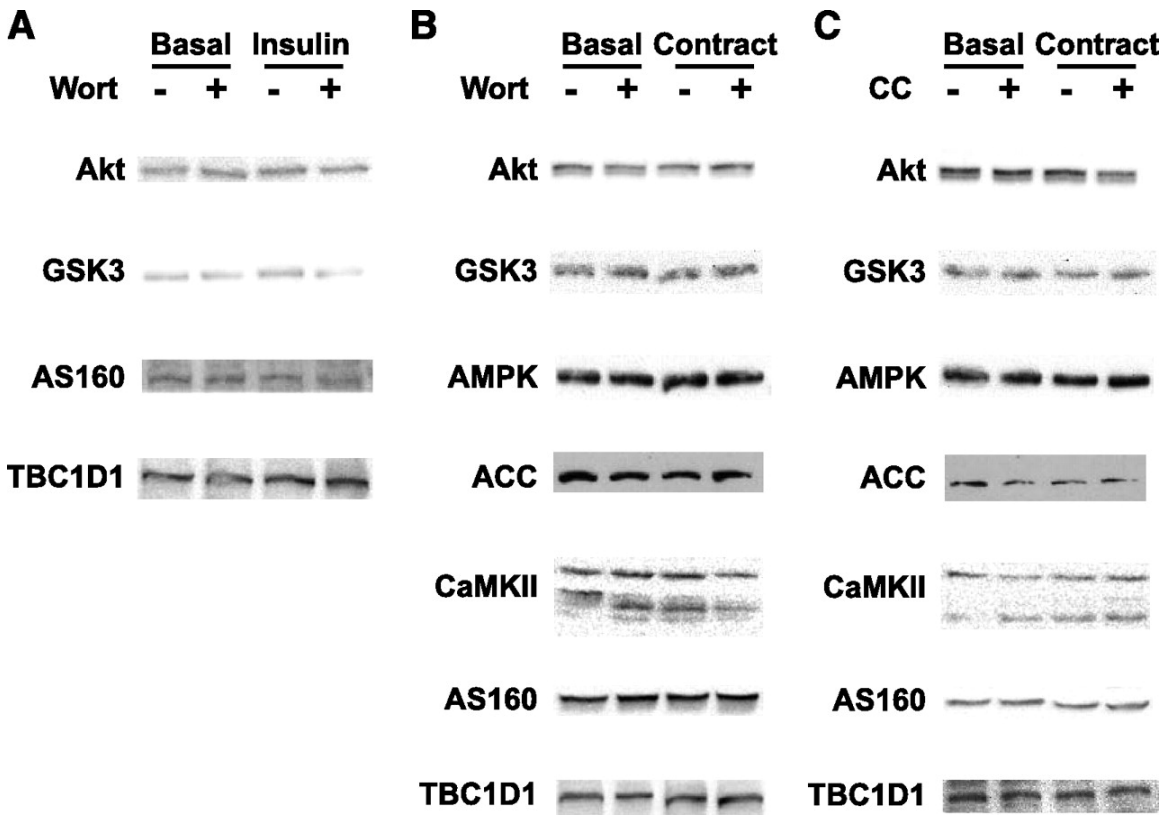

Figure 4.1
Abundance of total proteins (Akt, GSK3, AMPK, ACC, CaMKII, AS160 and TBC1D1. There were no statistically significant differences among groups (n=4/group) for total protein abundance in muscles with or without: (A) insulin and/or wortmannin; (B) contraction and/or wortmannin; and (C) contraction and/or Compound C. Wort: wortmannin. CC: Compound C.

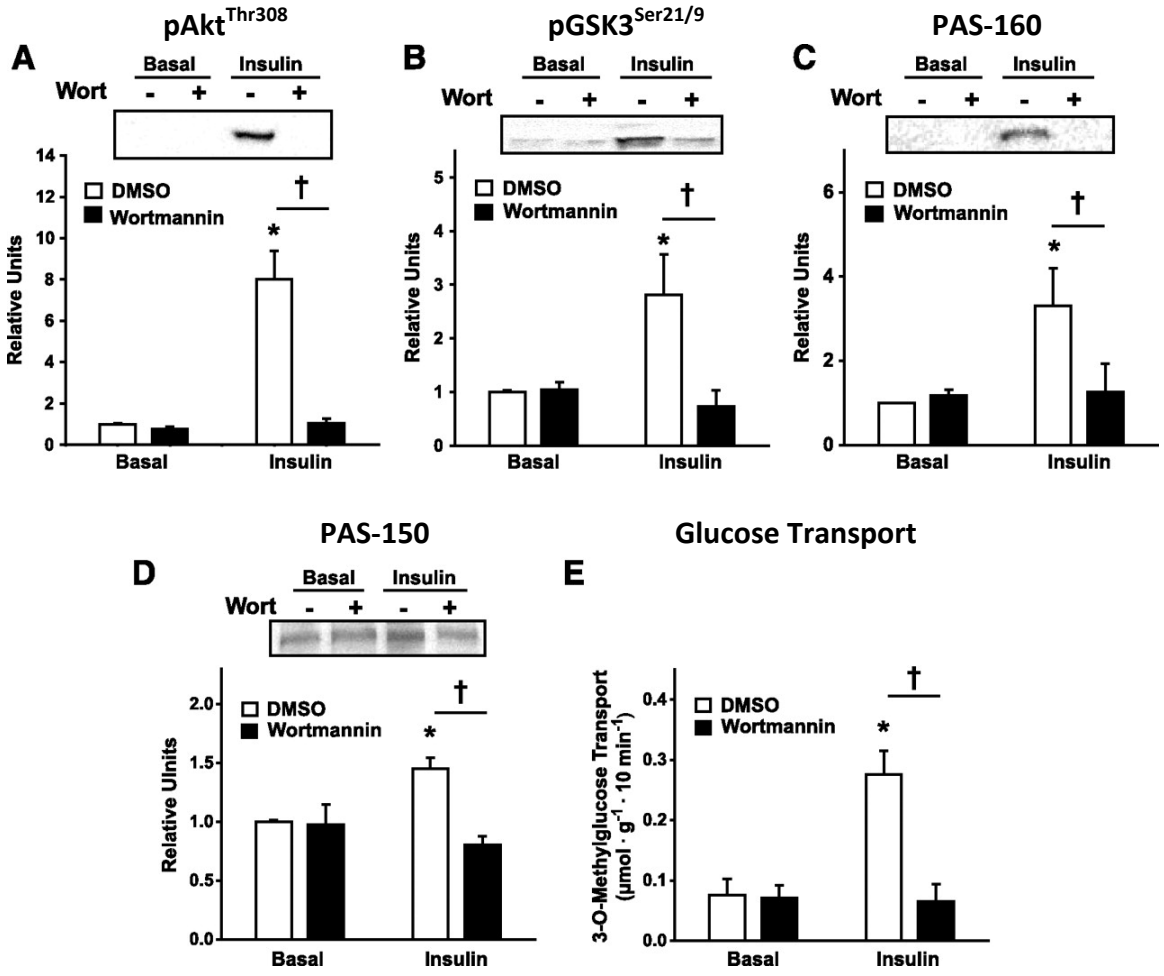

Figure 4.2
Effects of wortmannin on insulin-stimulated phosphorylation of Akt^{Thr308}, GSK3$^{Ser21/9}$, PAS-160, PAS-150 and glucose transport. (A) pAkt^{Thr308}, (B) pGSK3$^{Ser21/9}$, (C) PAS-160, (D) PAS-150 and (E) glucose transport. Paired isolated rat epitrochlearis muscles were incubated with or without 500 nM of wortmannin for 30 min. Muscles were then either incubated in identical media (Basal) or in solution that contained 2000 μU/mL of insulin for 20 min, freeze clamped immediately and used for immunoblotting or for 3-MG transport measurement. Data are means ± S.E., n = 5-9/group. Post hoc analysis: *, $P < 0.05$ (effect of insulin); †, $P < 0.05$ (effect of wortmannin). Wort: wortmannin. Open bars = DMSO; filled bars = wortmannin.

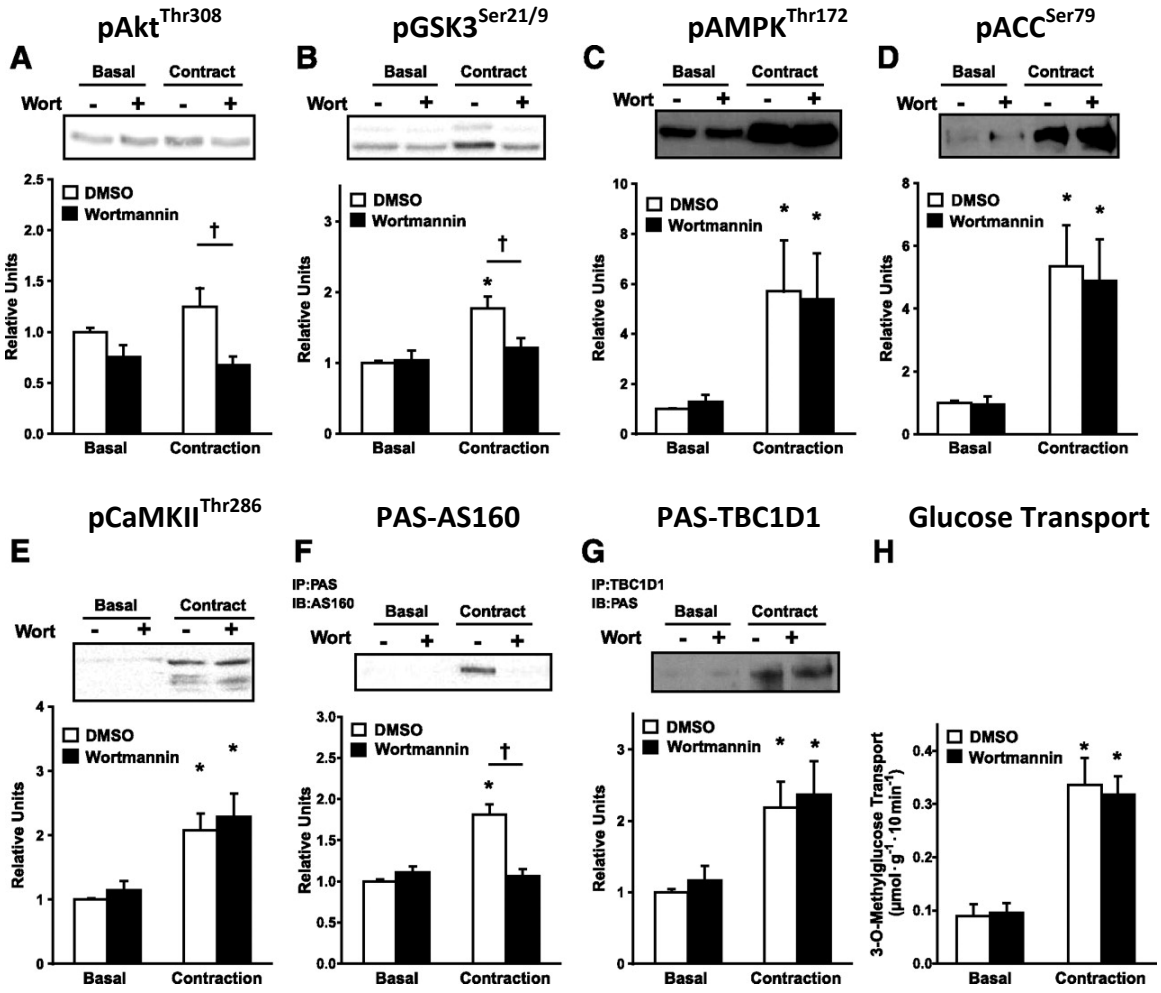

Figure 4.3

Effects of wortmannin on contraction-stimulated phosphorylation of Akt^{Thr308}, GSK3$^{Ser21/9}$, AMPK^{Thr172}, ACC^{Ser79}, CaMKII^{Thr286}, PAS-AS160, PAS-TBC1D1 and glucose transport. (A) pAkt^{Thr308}, (B) pGSK3$^{Ser21/9}$, (C) pAMPK^{Thr172}, (D) pACC^{Ser79}, (E) pCaMKII^{Thr286}, (F) PAS-AS160, (G) PAS-TBC1D1 and (H) glucose transport. Paired isolated rat epitrochlearis muscles were incubated with or without 500 nM of wortmannin for 30 min. Muscles were then either rested (Basal) or stimulated to contract for 20 min, freeze clamped immediately for immunoprecipitation and/or immunoblotting or used for 3-MG transport measurement. Data are means ± S.E., n = 9-17/group. Post hoc analysis: *, $P < 0.05$ (effect of contraction); †, $P < 0.05$ (effect of wortmannin). Wort: wortmannin. Open bars = DMSO; filled bars = wortmannin.

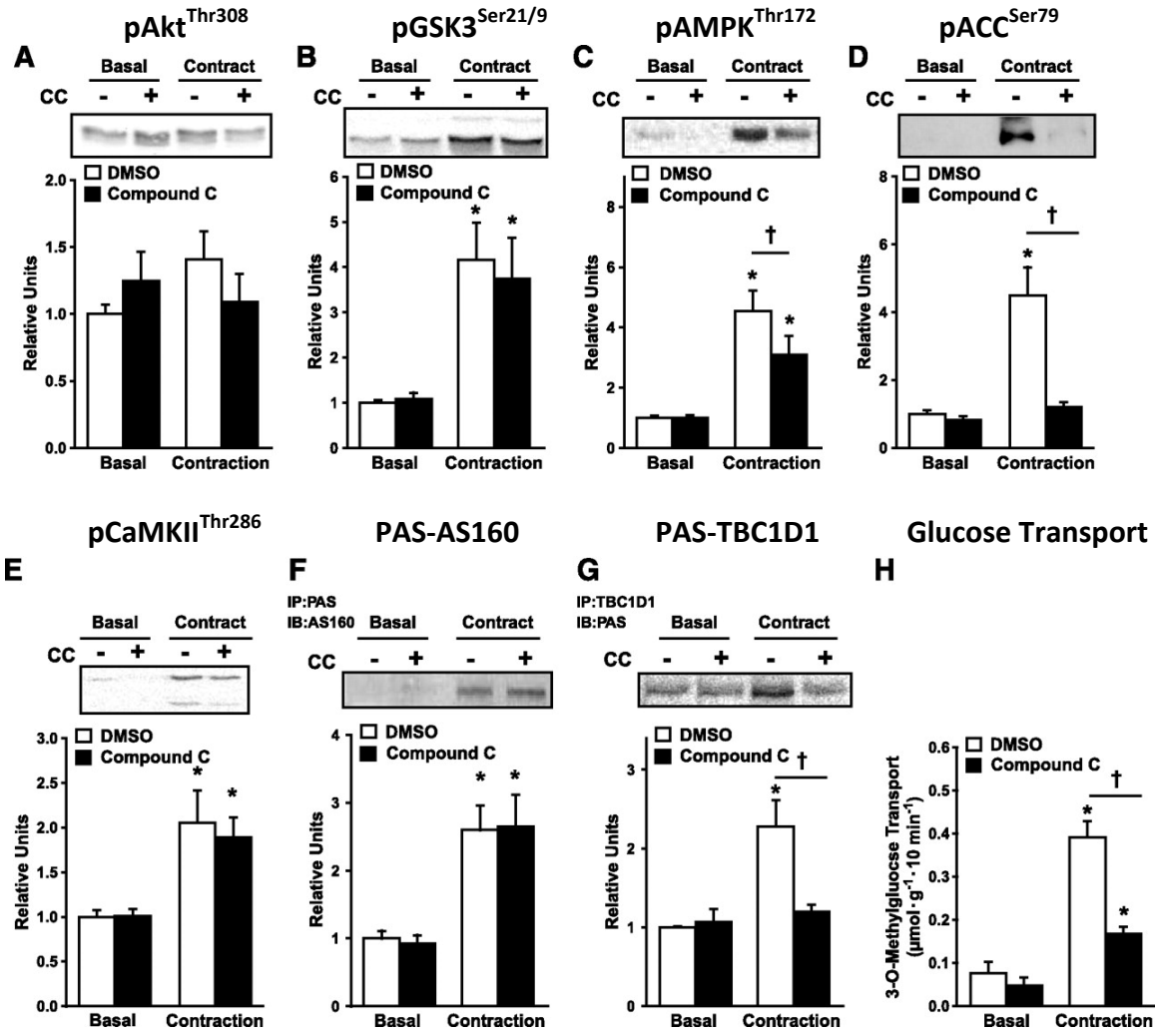

Figure 4.4
Effects of Compound C on contraction-stimulated phosphorylation of Akt^{Thr308}, GSK3$^{Ser21/9}$, AMPK^{Thr172}, ACC^{Ser79}, CaMKII^{Thr286}, PAS-AS160, PAS-TBC1D1 and glucose transport. (A) pAkt^{Thr308}, (B) pGSK3$^{Ser21/9}$, (C) pAMPK^{Thr172}, (D) pACC^{Ser79}, (E) pCaMKII^{Thr286}, (F) PAS-AS160, (G) PAS-TBC1D1 and (H) glucose transport. Paired isolated rat epitrochlearis muscles were incubated with or without 40 μM of Compound C for 60 min. Muscles were then either rested (Basal) or stimulated to contract for 20 min, freeze clamped immediately for immunoprecipitation and/or immunoblotting or used for 3-MG transport measurement. Data are means ± S.E., n = 6-14/group. Post hoc analysis: *, $P < 0.05$ (effect of contraction); †, $P < 0.05$ (effect of Compound C). CC: Compound C. Open bars = DMSO; filled bars = Compound C.

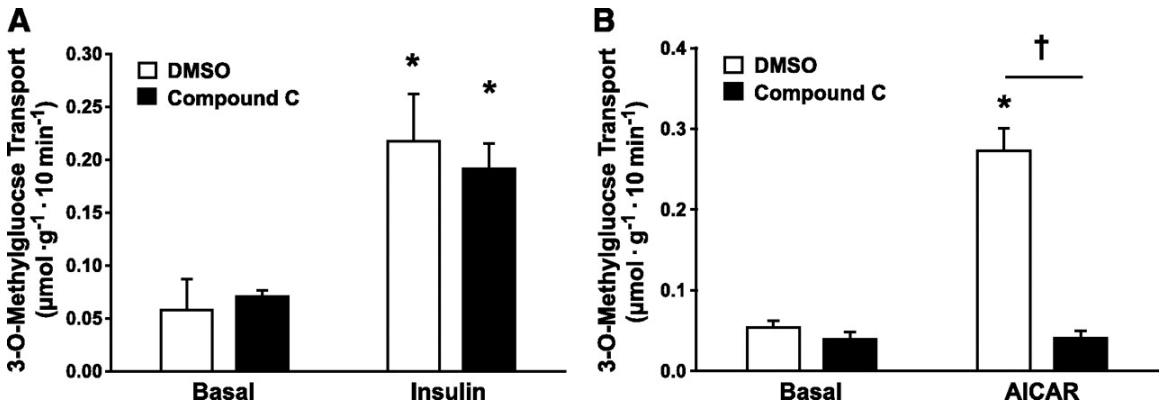

Figure 4.5
Effects of Compound C on insulin- and AICAR- stimulated glucose transport. (A) Insulin- and (B) AICAR- stimulated glucose transport. Paired isolated rat epitrochlearis muscles were incubated with or without 40 µM of Compound C for 60 min. Muscles were then either incubated in identical media (Basal) or (A) in solution that contained 2000 µU/mL of insulin for 20 min or (B) in solution that contained 2mM of AICAR for 40 min and used for 3-MG transport measurement. Data are means ± S.E., n = 6-14/group. Post hoc analysis: *, $P < 0.05$ (effect of insulin or AICAR); †, $P < 0.05$ (effect of Compound C). CC: Compound C. Open bars = DMSO; filled bars = Compound C.

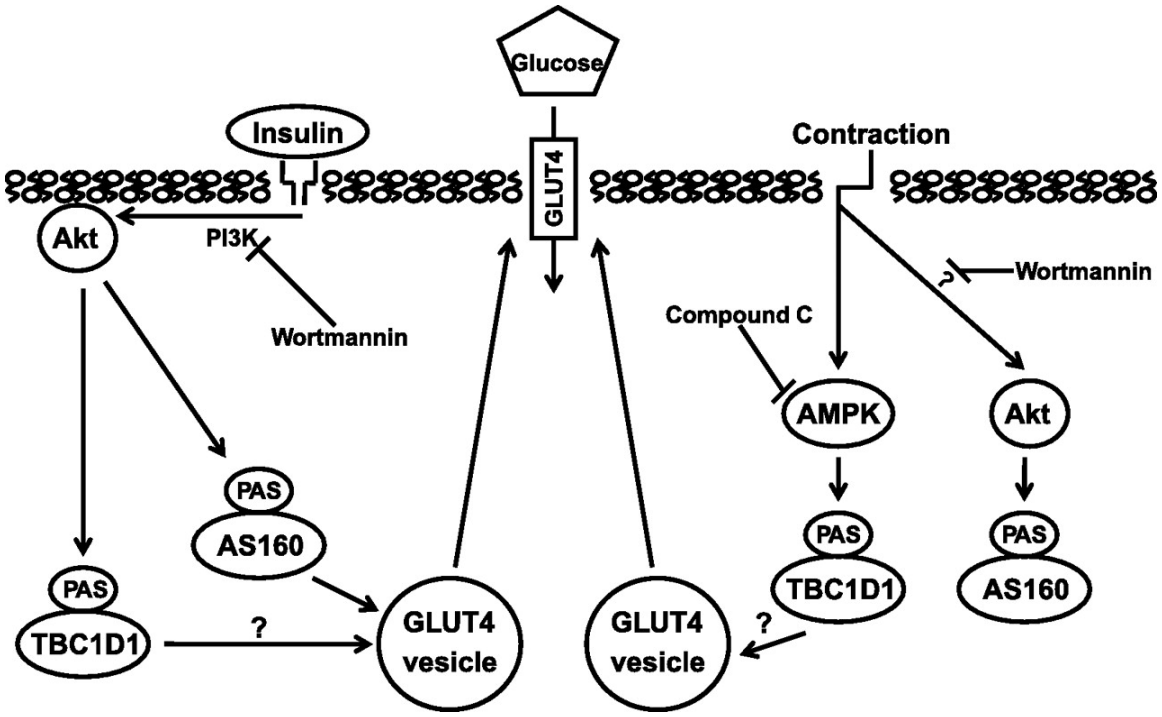

Figure 4.6
Working model for the roles of PAS-AS160 and PAS-TBC1D1 in insulin- and contraction-stimulated glucose transport. For clarity, the figure focuses on PAS-AS160 and PAS-TBC1D1 and does not depict other possible mechanisms which may influence glucose transport (e.g., calcium-mediated processes with contraction; binding of AS160 or TBC1D1 to 14-3-3 proteins, and/or phosphorylation on sites not recognized by anti-PAS with insulin or contraction). Insulin, by a phosphatidylinositol 3-kinase (PI3K) dependent mechanism, activates Akt, which phosphorylates Akt substrate of 160 kD (AS160 or TBC1D4) and TBC1D1 on sites identified using the phospho-Akt substrate (PAS) antibody. The PI3K inhibitor wortmannin completely eliminates insulin-stimulated glucose transport and PAS-phosphorylation of AS160 and TBC1D1. Increased PAS-AS160 is required for insulin's full effect on GLUT4 translocation and glucose transport. TBC1D1's role in insulin-stimulated glucose transport is uncertain. Contraction leads to increased phosphorylation of AMPK and Akt, although the ability of wortmannin to inhibit Akt activation by contraction suggests at PI3K-dependent process, the precise mechanism is unknown. AMPK-inhibition (by Compound C) completely eliminates the increased PAS-TBC1D1 without altering PAS-AS160, and wortmannin completely eliminates the increased PAS-AS160 without altering PAS-TBC1D1. Compound C partially inhibits contraction-stimulated glucose transport consistent with AMPK-related mechanisms accounting for a portion of contraction-mediated glucose transport. The concomitant AMPK-dependent inhibition of PAS-TBC1D1 suggests it may play a role in contraction-stimulated glucose transport.

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CHAPTER V

STUDY 3

Increased AS160 Phosphorylation, but Not TBC1D1 Phosphorylation, with Increased Post-exercise Insulin Sensitivity in Rat Skeletal Muscle

ABSTRACT

A single exercise bout can increase insulin-independent glucose transport immediately post-exercise and insulin-dependent glucose transport (GT) for several hours post-exercise. Akt substrate of 160 kDa (AS160) and TBC1D1 are paralog Rab GTPase activating proteins that have been proposed to contribute to these exercise effects. Previous research demonstrated greater AS160 and Akt threonine phosphorylation in rat skeletal muscle at 3-4 h post-exercise concomitant with enhanced insulin-stimulated GT. To further probe if these signaling events or TBC1D1 phosphorylation were important for the enhanced post-exercise insulin-stimulated GT, male Wistar rats were studied using four experimental protocols (2 h swim-exercise, differing with regard to timing of muscle sampling and whether food was provided post-exercise) that were known to vary in their influence of insulin-independent and insulin-dependent GT post-exercise. The results indicated that in isolated rat epitrochlearis muscle: 1) elevated phosphorylation of AS160 (measured using anti-phospho-Akt substrate, PAS-AS160, and phospho-specific anti Thr^{642}-AS160, pThr642-AS160) consistently tracked with elevated insulin-stimulated GT;

2) PAS-TBC1D1 was not different from sedentary values at 3 or 27 h post-exercise, when insulin sensitivity was increased; 3) insulin-stimulated Akt activity was not increased post-exercise in muscles with increased insulin sensitivity; 4) PAS-TBC1D1 was increased immediately post-exercise, when insulin-independent GT was elevated, and reversed at 3 and 27 h post-exercise, when insulin-independent GT was also reversed; and 5) there was no significant effect of exercise or insulin on total abundance of AS160, TBC1D1, Akt or GLUT4 protein with any of the protocols. The results are consistent with increased AS160 phosphorylation (PAS-AS160 or pThr642-AS160), but not increased PAS-TBC1D1 or Akt activity, being important for increased post-exercise insulin-stimulated GT in rat skeletal muscle. They also support the idea that increased TBC1D1 phosphorylation may play a role in the insulin-independent increase in GT post-exercise.

INTRODUCTION

A single bout of exercise leads to a subsequent increase in insulin-stimulated glucose transport (7, 9, 22, 40). The post-exercise increase in insulin sensitivity is attributable to increased insulin-stimulated GLUT4 cell surface localization (24). However, many studies have found that prior exercise has no effect on proximal insulin signaling steps, including: insulin receptor binding (4, 5, 55), insulin receptor tyrosine phosphorylation (24, 26, 47, 53), insulin receptor tyrosine kinase activity (46, 47, 52), insulin receptor substrate tyrosine phosphorylation (24, 26, 54), insulin receptor substrate associated phosphatidylinositol-3-kinase (PI3K) activity (46, 53), and Akt serine phosphorylation (1, 16, 23, 46, 52).

The Rab GTPase activating protein (Rab GAP) Akt substrate of 160 kDa (AS160; also known as TBC1D4) is the most distal signaling protein that has been implicated in insulin-mediated GLUT4 translocation (8, 32, 42, 43). Arias et al. (1) found that phosphorylation of AS160 is greater in insulin-stimulated muscles from exercised (3-4 h post-exercise) rats compared with sedentary controls. However, this increase was not because of greater insulin sensitivity for AS160 phosphorylation, rather it was because the baseline phosphorylation of AS160 in the absence of insulin remained increased 3-4 h post-exercise. In other words, basal AS160 phosphorylation remained higher at 3-4 h post-exercise compared to resting, and this elevated baseline accounted for the greater AS160 phosphorylation found in muscles that were subsequently stimulated with insulin. This observation is also supported by results in humans that indicated that skeletal muscle AS160 phosphorylation can remain elevated for 2 to 14 h post-exercise without elevated insulin (17, 44, 49). Arias et al. (1) proposed that the persistent increase in insulin-stimulated glucose transport is attributable, at least in part, to the sustained increase in AS160 phosphorylation after acute exercise.

Phosphorylation of TBC1D1, a paralog RabGAP of AS160, may also regulate GLUT4 translocation (10, 28, 41). In skeletal muscle, TBC1D1 phosphorylation is increased in response to insulin (19, 45), contraction (3, 18, 19, 45) or incubation with the AMP-activated protein kinase (AMPK) activator AICAR (45). However, it is unknown if TBC1D1 phosphorylation is increased in response to in vivo exercise. The relationship between TBC1D1 phosphorylation and the post-exercise increase in insulin-stimulated glucose transport has also not been investigated. Experiments using 3T3-L1 (10) or L6 (28) cells suggest that although insulin can induce TBC1D1 phosphorylation, TBC1D1's

inhibitory effects on GLUT4 translocation may not be subject to regulation by insulin. It remains possible that TBC1D1 participates in GLUT4 trafficking induced by insulin-independent stimuli, e.g., in vivo exercise.

Consistent with previous reports (12, 16, 23, 46, 52), Arias et al. (1) found that insulin-stimulated Akt serine phosphorylation (pSerAkt) was not enhanced at 3-4 h post-exercise. However, in the same muscles, insulin-stimulated Akt threonine phosphorylation (pThrAkt) was greater at 3-4 h post-exercise compared to sedentary controls. The insulin-stimulated phosphorylation of two Akt substrates (glycogen synthase kinase-3, GSK3, and AS160) was not greater at 3-4 hr post-exercise, suggesting that greater insulin-stimulated pThrAkt after exercise did not result in greater Akt activity. Nonetheless, it would be important to determine if this prediction is true because enhanced insulin-stimulated Akt activity could potentially contribute to increase insulin sensitivity.

To further probe the possible functional importance of AS160, TBC1D1 and Akt for the post-exercise increase in insulin-stimulated glucose transport, we used four experimental protocols (differing with regard to the timing of muscle sampling and whether food was provided post-exercise) that were known to vary in their influence on insulin-independent and insulin-dependent glucose transport after exercise (male Wistar rats, 2 h swim-exercise). We hypothesized that the protocols with enhanced insulin-dependent glucose transport after exercise would be accompanied by increased AS160 phosphorylation, and protocols without enhanced insulin-dependent glucose transport after exercise would not be characterized by elevated AS160 phosphorylation. We also hypothesized that increased TBC1D1 phosphorylation for exercised versus sedentary

groups would be found in protocols with elevated insulin-independent glucose transport, but not in protocols with an exercise effect on insulin-dependent glucose transport. We further hypothesized that the increased pThrAkt found in insulin-stimulated skeletal muscle at 3 h post-exercise would not be accompanied by an increase in Akt activity compared to sedentary controls.

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 from Pierce Biotechnology (Rockford, IL). Anti-Akt (no. 9272), anti-phospho-Thr^{308}Akt (pThrAkt, no. 9275), anti-phospho-(Ser/Thr) Akt substrate (PAS, no. 9611), anti-GLUT4 (no. 2299) and goat anti-rabbit IgG HRP conjugate (no. 7074) were from Cell Signaling Technology (Danvers, MA). PAS was designed to recognize Akt phosphorylation motif peptide sequences (RXRXXpT/S). TBC1D1 polyclonal antibody was provided by Dr. Makoto Kanzaki at Tohoku University (35). Anti-AS160 (no. 07-741), anti-phospho-Thr^{642}AS160 (pThrAS160, no. 07-802), protein G agarose beads (no. 16-266) and Akt immunoprecipitation kinase assay kit (no. 17-188) were from Upstate USA (Charlottesville, VA). 3-*O*-Methyl-[^{3}H]glucose ([^{3}H]3-MG) was from Sigma-Aldrich. [^{14}C]Mannitol and [γ-^{32}P]ATP were from Perkin Elmer (Waltham, MA). Other reagents were 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. Male Wistar rats (~140–200 g; Harlan, Indianapolis, IN) were studied using four experimental protocols. In each protocol, rats were provided with rodent chow (Lab Diet; PMI Nutritional International, Brentwood, MO) and water ad libitum. At 1700 on the night before the experiments, rats were housed individually and provided ad libitum water and 4 g of chow each.

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 (6 or 7 rats per barrel) for 4 x 30 min bouts with a 5 min rest period between each bout. The 4 experimental protocols differed for the PEX groups and their respective SED controls only with regard to: 1) the time after completion of exercise when muscles were dissected out from anesthetized rats, and 2) whether rats had access to chow after exercise. The 8 groups (a PEX and a SED group for each protocol) were: 1) anesthetized immediately post-exercise without access to food after exercise (0hPEX and 0hSED); 2) anesthetized 3 h post-exercise without access to food after exercise (3hPEX and 3hSED); 3) anesthetized 3 h post-exercise with unlimited access to food after exercise (3hPEX-Chow and 3hSED-Chow); and 4) anesthetized 27 h post-exercise without access to food after exercise (27hPEX and 27hSED). While 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 all incubation steps, flasks were continuously gassed from above with 95 % O_2/5 % CO_2 and shaken in a heated water bath. Isolated

epitrochlearis muscles were incubated in Krebs-Henseleit buffer (KHB) + 0.1 % bovine serum albumin (BSA) + 8 mM glucose + 2mM 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. Insulin remained present at the same concentration throughout all subsequent incubations. After the initial incubation, muscles were transferred to vials containing KHB + BSA + 2 mM pyruvate + 6 mM mannitol (Solution 2) at 30°C for 10 min. Finally, muscles were transferred to flasks containing KHB, 0.1 % BSA with 8 mM 3-MG (including [^{3}H]3-MG 0.25 mCi/mmol), and 2 mM mannitol (including [^{14}C]mannitol 0.1 mCi/mmol). After incubation with 3-MG for 15 min, the muscles were rapidly blotted on filter paper dampened with incubation media, trimmed, freeze-clamped, and stored at –80°C until processed as described below.

3-MG transport and protein phosphorylation. The procedures for 3-MG transport, immunoprecipitation and immunoblotting were conducted as previously described (19). For PAS-AS160, muscles were immunoprecipitated using anti-PAS, followed by immunoblotting by anti-AS160. For PAS-TBC1D1, muscles were immunoprecipitated using anti-TBC1D1, followed by immunoblotting by anti-PAS. The mean values for basal muscles (sedentary without insulin) on each blot were normalized to equal 1.0, and then all samples on the blot were expressed relative to the normalized basal value.

Akt activity measurement. Akt activity was determined according to the manufacturer's instructions (Upstate USA, Charlottesville, VA). Briefly, protein G-agarose beads were rotated overnight with anti-Akt/PH domain clone SKB1 (no. 05-591). The antibody/protein G-agarose mixture was combined with 300 μg of protein from each

sample and rotated for 2 h at 4°C. The antigen/antibody/protein G-agarose complex was combined with Akt substrate peptide (no. 12-340) and [γ-^{32}P]ATP (final concentration of 1μCi/μl) and shaken at room temperature for 60 min. Next, the complex was centrifuged (4,000 g for 1 min) and 40 μl of supernatant was collected and transferred to phosphocellulose paper. After washes (3 times with 1.5% phosphoric acid and once with acetone), the phosphocellulose paper was transferred to a vial containing scintillation cocktail for scintillation counting. The mean values for basal muscles (sedentary without insulin) on each experiment day were normalized to equal 1.0, and then all samples were expressed relative to the normalized basal value.

Muscle glycogen concentration. Muscles used for measurement of glycogen were frozen immediately after dissection, weighed and homogenized in ice-cold 0.3M perchloric acid. An aliquot of the homogenate was stored at -80°C for later determination of glycogen concentration by the amyloglucosidase method (37).

Statistical analysis. Statistical analyses were performed using Sigma Stat version 2.0 (San Rafael, CA). Data are expressed as mean ±SE. $P \leq 0.05$ was considered statistically significant. Data from the 3hPEX and 27hPEX experiments were analyzed with two-way ANOVA and the Student-Newman-Keuls post-hoc test. When data failed the Levene Median test for equal variance, the Kruskal-Wallis nonparametric ANOVA on ranks was used with Dunn's post-hoc test. The insulin-stimulated increases (Δ-insulin) in glucose transport, protein phosphorylation and Akt activity were calculated by subtracting the values for muscles incubated without insulin from the respective values of paired muscles incubated with insulin. Student's t-test was used for the analysis of

glucose transport and protein phosphorylation for 0hPEX experiments and Δ-insulin values for 3hPEX and 27hPEX experiments.

RESULTS

3-MG transport. Insulin-independent glucose transport was approximately 2.3-fold greater ($P < 0.05$) for 0hPEX rats compared with sedentary (0hSED) controls (Fig. 5.1A), and this increase was completely reversed at both 3 and 27 h post-exercise (Fig. 5.1B-D). Insulin-treated muscles had a greater glucose transport than paired muscles incubated without insulin for all groups (Fig. 5.1B-D). PEX versus SED groups that were not refed chow after exercise (Fig. 5.1B and 5.1D) had significantly greater glucose transport for insulin-stimulated muscles at both 3hPEX ($P < 0.001$) and 27hPEX ($P < 0.01$). Significant ($P < 0.01$) effects of prior exercise were also found for Δ-insulin glucose transport for the 3hPEX (0.322 ± 0.052 $\mu mol \cdot g^{-1} \cdot 15min^{-1}$) versus 3hSED ($0.125 \pm 0.024$ $\mu mol \cdot g^{-1} \cdot 15min^{-1}$) and 27hPEX ($0.523 \pm 0.075$ $\mu mol \cdot g^{-1} \cdot 15min^{-1}$) versus 27hSED ($0.240 \pm 0.068$ $\mu mol \cdot g^{-1} \cdot 15min^{-1}$) groups. The exercise effects on glucose transport by insulin-stimulated muscles and Δ-insulin glucose transport were lost with chow refeeding (Fig. 5.1C), i.e. there were no significant differences between the 3hSED-Chow (0.144 ± 0.039 $\mu mol \cdot g^{-1} \cdot 15min^{-1}$) and 3hPEX-Chow ($0.187 \pm 0.037$ $\mu mol \cdot g^{-1} \cdot 15min^{-1}$) groups.

Total protein abundance. The total protein abundance of AS160 (Fig. 5.2, *A–D*), TBC1D1 (Fig. 5.2, *E–H*), GLUT4 (data not shown), and Akt (data not shown) was unaltered by the experimental treatments (insulin or exercise; $n = 4$ for each protein within every exercise and insulin treatment group) in every protocol studied (0-h PEX, 3-h PEX, 3-h PEX-chow, or 27-h PEX).

AS160 phosphorylation. Insulin treatment compared to no insulin resulted in significantly greater PAS-phosphorylation of AS160 (PAS-AS160) in all groups (Fig. 5.3A-C). Consistent with our previous study (1), PAS-AS160 in muscles incubated without insulin was significantly greater ($P < 0.05$) in the 3hPEX compared to the 3hSED group (Fig. 5.3A). Also consistent with our previous study (1), PAS-AS160 in muscles incubated with insulin was significantly greater ($P < 0.05$) in the 3hPEX compared to the 3hSED group (Fig. 5.3A). In contrast, PAS-AS160 was not different between 3hSED-Chow and 3hPEX-Chow groups, with or without insulin during the incubations (Fig. 5.3B). PAS-AS160 in muscles incubated without insulin was significantly greater ($P < 0.05$) in the 27hPEX compared to the 27hSED group (Fig. 5.3C). PAS-AS160 in muscles incubated with insulin was also significantly greater ($P < 0.05$) in 27hPEX compared to 27hPEX group (Fig. 5.3C). The Δ-insulin values were not significantly different between SED and PEX rats for any of the groups (data not shown).

AS160 Thr642 phosphorylation. AS160 Thr642 phosphorylation was significantly elevated ($P < 0.05$) for the 0hPEX compared to the 0hSED group (Fig. 5.4A). Insulin treatment compared to no insulin resulted in significantly greater pThr642-AS160 in all groups (Fig. 5.4B-D). Consistent with the results for PAS-AS160, pThr642-AS160 in muscles incubated without insulin was significantly greater ($P < 0.001$) in the 3hPEX compared to the 3hSED group (Fig. 5.4B). Also consistent with the results on PAS-AS160, pThr642-AS160 in muscles incubated with insulin was significantly greater ($P < 0.001$) in the 3hPEX compared to the 3hSED group (Fig. 5.4B). As was found with PAS-AS160, pThr642-AS160 was not different between 3hSED-Chow and 3hPEX-Chow groups (Fig. 5.4C). The 27hPEX group compared to the

27hSED group had greater pThr642-AS160 with ($P < 0.05$) or without ($P < 0.005$) insulin (Fig. 5.4D). The Δ-insulin values were not significantly different between SED and PEX rats for any of the protocols (data not shown).

TBC1D1 phosphorylation. PAS-phosphorylation of TBC1D1 (PAS-TBC1D1) was significantly elevated ($P < 0.001$) in 0hPEX compared to 0hSED rats (Fig. 5.5A). This increase in was completely reversed at 3 h post-exercise, with or without refeeding, and at 27 h post-exercise (Fig. 5.5B-D). Insulin treatment compared to no insulin resulted in significantly greater PAS-TBC1D1 in all groups (Fig. 5.5B-D). However, in contrast to PAS-AS160, PAS-TBC1D1 was not different for at 3hPEX, 3hPEX-Chow and 27hPEX compared to the respective sedentary controls (3hSED, 3hSED-Chow and 27hSED) regardless of insulin concentration. The Δ-insulin values were also not significantly different between SED and PEX rats for any of the groups (data not shown).

Akt threonine phosphorylation. Insulin treatment compared to no insulin resulted in significantly greater pThrAkt in all groups (Fig. 5.6A-C). Consistent with our previous results (1), pThrAkt in muscles incubated with insulin was greater ($P < 0.05$) for the 3hPEX group compared with 3hSED controls (Fig. 5.6A). Furthermore, there was also a significant ($P < 0.05$) increase for the 27hPEX versus 27hSED group (Fig. 5.6C). The Δ-insulin values were significantly greater for 3hPEX versus 3hSED and 27hPEX versus 27hSED groups ($P < 0.05$). In contrast, neither the pThrAkt in muscles incubated with insulin (Fig. 5.6B) nor the Δ-insulin values (data not shown) were significantly different between 3hSED-Chow and 3hPEX-Chow groups.

Akt activity. Insulin treatment compared to no insulin resulted in significantly greater Akt activity (Fig. 5.6D). Unlike pThrAkt, Akt activity for insulin-stimulated

muscles was not significantly different between the 3hSED and 3hPEX groups. The Δ-insulin values were also not different between 3hSED (0.831 ± 0.173 relative units) and 3hPEX rats (0.886 ± 0.373 relative units).

Glycogen concentration. Epitrochlearis glycogen concentration (Table 5.1) was reduced by ~60% ($P < 0.001$) immediately post-exercise (0hPEX vs. 0hSED). In the exercised groups that were not refed (3hPEX and 27hPEX), glycogen did not increase significantly above the 0hPEX values. Glycogen values were also essentially unchanged for 3hSED versus 0hSED, but there was an ~30% reduction ($P < 0.05$) for the 27hSED group compared to the other sedentary groups that were not refed (0hSED and 3hSED). With this decline there was no significant difference for the 27hSED compared to any of the exercised groups that were not refed (0hPEX, 3hPEX and 27hPEX). Glycogen, which was increased in the 3hPEX-Chow above all other groups ($P < 0.05$), was ~4-fold greater than the 0hPEX group and ~50% greater than the 3hSED-Chow rats. Chow refeeding did not significantly increase glycogen for the 3hSED group compared to the 0hSED or 3hSED animals.

DISCUSSION

The results support the hypothesis that a sustained increase in AS160 phosphorylation, but not TBC1D1 phosphorylation, consistently occurs with protocols that cause a sustained post-exercise increase in insulin-stimulated glucose transport. Specifically, the data: 1) indicated an increased PAS-AS160 and pThr642-AS160 in skeletal muscle, with or without insulin, and enhanced insulin-stimulated glucose transport at 3 h post-exercise in rats that were not refed; 2) extended the previously

published results (1) by demonstrating the post-exercise increase in PAS-AS160 and pThr642-AS160 together with increased insulin-stimulated glucose transport at 27 h post-exercise in rats not refed; 3) demonstrated the elimination of increased PAS-AS160, pThr642-AS160 and increased insulin-stimulated glucose transport at 3 h post-exercise in rats that were refed; and 4) found that neither insulin nor exercise altered the total abundance of AS160, TBC1D1, Akt or GLUT4 protein in any of the protocols tested. In addition, there was not a persistent increase in PAS-TBC1D1 at 3 or 27 h post-exercise, supporting the idea that AS160 phosphorylation, rather than TBC1D1 phosphorylation, is important for improved insulin sensitivity after exercise. Furthermore, the lack of an increase in insulin-stimulated Akt activity after exercise indicates that this mechanism cannot account for the improved insulin sensitivity.

There was a persistent increase in basal (without insulin) AS160 phosphorylation in the 3hPEX and 27hPEX groups, and this higher baseline value accounted for the greater AS160 phosphorylation in insulin-stimulated muscles after exercise. What is a possible mechanism that could link the elevated AS160 phosphorylation without insulin with increased insulin-stimulated glucose transport after exercise? It is important to recognize that AS160 does not regulate all of the steps required for GLUT4 to be redistributed from intracellular storage vesicles to the cell surface membranes where they are able to facilitate glucose uptake (27, 51). Studies in both 3T3-L1 adipocytes (2) and L6 myoblasts (38) suggest that AS160 phosphorylation is required for insulin-stimulated docking of GLUT4 vesicles to cell surface membranes. AS160 apparently does not regulate several of the other steps that are required for complete GLUT4 translocation and increased glucose transport rate, including the recruitment, tethering, and fusion of

GLUT4 vesicles with surface membranes and possibly GLUT4 activation (2, 20, 27, 51). We speculate that the persistent increase in AS160 phosphorylation in the absence of insulin results in a change in the localization of a portion of the GLUT4 vesicles (perhaps there is a persistent increase in docked vesicles) so that they are more susceptible to the insulin-stimulated, AS160-independent steps of GLUT4 vesicle traffic. The idea is that the persistent increase in AS160 phosphorylation found several hours after exercise may serve to "prime the pump" so that, when the muscle is subsequently stimulated with insulin, there is a greater pool of GLUT4 that is susceptible to being recruited by a given amount of insulin. This scenario is similar to a mechanism that was previously proposed by John Holloszy (25). We propose that AS160-independent regulatory steps which become activated upon insulin stimulation, acting in concert with the persistent effect of exercise on AS160 phosphorylation, culminate in the enhanced post-exercise insulin-stimulated GLUT4 translocation and glucose transport. This model does not exclude the possibility that prior exercise also amplifies insulin signaling steps other than AS160 phosphorylation.

In contrast to PAS-AS160 and pThr642-AS160, PAS-TBC1D1 was not different for the 3hPEX, 3hPEX-Chow and 27hPEX groups compared to their respective sedentary control groups (3hSED, 3hSED-Chow and 27hSED), demonstrating that enhanced PAS-TBC1D1 is not essential for the post-exercise increase in insulin-stimulated glucose transport. It remains possible that there is a persistent effect of exercise on TBC1D1 by another mechanism such as phosphorylation on sites not recognized by the PAS antibody or changes in subcellular localization (10, 11, 41). Therefore, we cannot rule out the possibility that TBC1D1 participates in the increased insulin-stimulated glucose transport

after exercise. Nevertheless, the results suggest that: 1) PAS-phosphorylation of TBC1D1 is not essential for increased insulin-stimulated glucose transport post-exercise, and 2) post-exercise reversal of the increased phosphorylation of TBC1D1 and AS160 are regulated differently.

We confirmed the previous observation indicating that there is an increase in insulin-stimulated pThrAkt at 3 h post-exercise in rats not fed after exercise (1), and extended this result to show enhanced insulin-stimulated pThrAkt at 27 h post-exercise in rats that remained unfed. Furthermore, insulin-stimulated pThrAkt was not increased 3 h post-exercise in rats that were refed. Thus, the effect of exercise on insulin-stimulated pThrAkt tracked with insulin-stimulated glucose transport for each protocols. However, because there was not a concomitant increase in insulin-stimulated Akt activity at 3 h post-exercise in rats not refed, it seems unlikely that the elevated pThrAkt could account for improved insulin sensitivity. These data suggest that in post-exercise muscles: 1) the post-exercise enhancement of insulin-stimulated pThrAkt does not induce greater than usual increase in insulin-stimulated Akt activity, and 2) enhanced Akt activity is not responsible for the enhanced insulin-stimulated glucose transport post-exercise. The similar Akt activity found in sedentary compared to post-exercise groups is also consistent with our previous results indicating that exercise did not alter the insulin-stimulated (Δ-insulin) increase in phosphorylation of Akt substrates, pGSK3 and PAS-AS160 (1). The current study also found that prior exercise did not alter the ability of insulin to induce the phosphorylation of Akt substrates (as assessed by PAS-AS160, pThr642-AS160 and PAS-TBC1D1) in skeletal muscle. These results are consistent with

a number of studies indicating that acute exercise does not amplify proximal insulin signaling in skeletal muscle.

Although elevated PAS-TBC1D1 could not explain the long-lasting increase in insulin-dependent glucose transport found at 3 or 27 h post-exercise, the increased PAS-TBC1D1 tracked closely with the more transient, post-exercise effect on insulin-independent glucose transport (14, 50). The exercise effects on PAS-TBC1D1 and glucose transport in the absence of insulin were both substantially elevated at 0hPEX, and the exercise effects in both were also completely reversed in the 3hPEX, 3hPEX-Chow and 27hPEX groups. This consistent association supports the idea that PAS-TBC1D1 may play an important role in contraction-stimulated glucose transport (3, 19). The relationship between PAS-TBC1D1 and insulin-independent glucose transport with contraction or exercise is in striking contrast to the results for PAS-AS160 which remained increased without insulin at 3hPEX and 27hPEX compared to respective sedentary controls in the absence of an exercise effect on insulin-independent glucose transport. We previously found that wortmannin completely eliminated the contraction-stimulated increase in PAS-AS160 without altering the contraction-stimulated increases in PAS-TBC1D1 or glucose transport (19). Furthermore, an AMPK inhibitor eliminated the contraction effect on PAS-TBC1D1 and reduced contraction-stimulated glucose transport by 65% without attenuating the contraction-induced increase in PAS-AS160. In conjunction with many other results (1, 6, 15, 19, 48), these data suggested that AS160 phosphorylation is neither necessary nor sufficient for increased insulin-independent glucose transport after exercise or contraction. The results are also consistent with the possibility that TBC1D1 plays a role in a portion of the insulin-independent increase in

glucose transport induced by exercise or contraction. Our working hypothesis is that AS160 phosphorylation is more important for insulin-stimulated glucose transport, whereas TBC1D1 phosphorylation is more important for exercise-stimulated glucose transport. Regulation of AS160 by its calmodulin binding domain (CBD) has been implicated in a portion of the contraction-stimulated increase in glucose transport (31). TBC1D1 also has a CBD, but its role in contraction-stimulated glucose transport has not been assessed.

Both AS160 and TBC1D1 can be phosphorylated by multiple kinases (11, 21). Many studies have shown that in vivo exercise can activate AMPK (23, 39, 44, 48), including Arias et al. (1) who found increased pAMPK at 0hPEX using the same protocol as the current study. Although increased Akt activity after in vivo exercise has been reported (44, 48), some studies have not detected Akt activation in skeletal muscle with in vivo exercise (17, 33, 53), including Arias et al. (1) who found unaltered pThrAkt and pSerAkt at 0hPEX. These results suggest AMPK as a candidate for the increased AS160 and TBC1D1 phosphorylation at 0hPEX, but we cannot rule out the possibility that Akt was transiently activated in the early stages of the exercise or a role for other kinases that were not tested.

Both AS160 and TBC1D1 can be phosphorylated on multiple sites (11, 21, 29). Treebak et al. (49) recently reported that after one-legged exercise by humans resulted in an elevation of AS160 phosphorylation on S318, S341, and S751, with a trend for an increase on S588 in muscle in the absence of insulin infusion (at 240 min post-exercise) and with insulin infusion (at 340 min post-exercise). There was no effect of prior exercise on AS160 phosphorylation detected on T642, S666, or using anti-PAS. Previous

research with humans had found increased PAS-AS160 at 2.5 h post-exercise (44). Although the explanation for the different results for PAS-AS160 and pThr642-AS160 after exercise is uncertain, there is a great deal of evidence for a persistent increase in AS160 in skeletal muscle after acute exercise.

As expected, exercise resulted in decreased muscle glycogen content. It has been hypothesized that reduced glycogen concentration is involved in the postexercise increase in insulin-stimulated glucose transport (13, 34, 36). However, activation of AMPK by AICAR can result in subsequently elevated insulin-stimulated glucose transport in the absence of altered glycogen levels (16). Furthermore, a study that compared multiple in situ contraction protocols found that all protocols that resulted in decreased glycogen concentration also resulted in greater insulin sensitivity postexercise, but not all protocols that induced a similar decrement in glycogen levels were also characterized by improved insulin sensitivity (30). These findings suggest that glycogen reduction may be necessary, but not sufficient, for a postexercise-induced increase in insulin-stimulated glucose transport. The reduced muscle glycogen levels at the 3-h PEX and 27-h PEX groups compared with the 0-h SED group are consistent with the idea that glycogen reduction may contribute to the postexercise increase in insulin-stimulated glucose transport, and the higher glycogen content in 3-h PEX-chow group vs. the 3-h SED group is consistent with the idea that glycogen resynthesis may contribute to the reversal of the postexercise effect on insulin sensitivity. However, the lack of difference in glycogen content between 27-h SED and 27-h PEX groups, taken together with earlier results (30), suggests that lower glycogen values do not fully explain the mechanism for the prolonged increase in insulin sensitivity on the day after exercise.

In conclusion, the results of this study support a model in which AS160 and TBC1D1, two Rab GAP paralogs, have different roles in the post-exercise effects on skeletal muscle glucose transport. The major, new findings indicate that: 1) elevated PAS-AS160 or pThr642-AS160 after exercise consistently tracked with elevated insulin-stimulated glucose transport, supporting the idea that AS160 phosphorylation plays a role in the post-exercise increase in insulin sensitivity; 2) PAS-TBC1D1 was not different from sedentary control values at 3 or 27 h after exercise, when insulin-stimulated glucose transport was increased, demonstrating that the post-exercise dephosphorylation of TBC1D1 is regulated differently from AS160, and PAS-TBC1D1 is not important for the post-exercise increase in insulin-stimulated glucose transport; 3) insulin-stimulated Akt activity was not increased at 3 h after exercise, at which time insulin-stimulated glucose transport was enhanced, demonstrating that it cannot account for increased post-exercise insulin sensitivity; and 4) the temporal relationship between the post-exercise effects on insulin-independent glucose transport and PAS-TBC1D1 (both elevated immediately after in vivo exercise, and both reversed at 3 and 27 h post-exercise) supports the hypothesis that TBC1D1 phosphorylation may play a role in the transient exercise-induced increase in glucose transport in the absence of insulin. It will be important for future research to identify the unique phospho-signatures of AS160 and TBC1D1 in response to exercise and insulin, to determine their protein-protein interactions (especially with 14-3-3 proteins), and to characterize how their subcellular localization is influenced by exercise and insulin.

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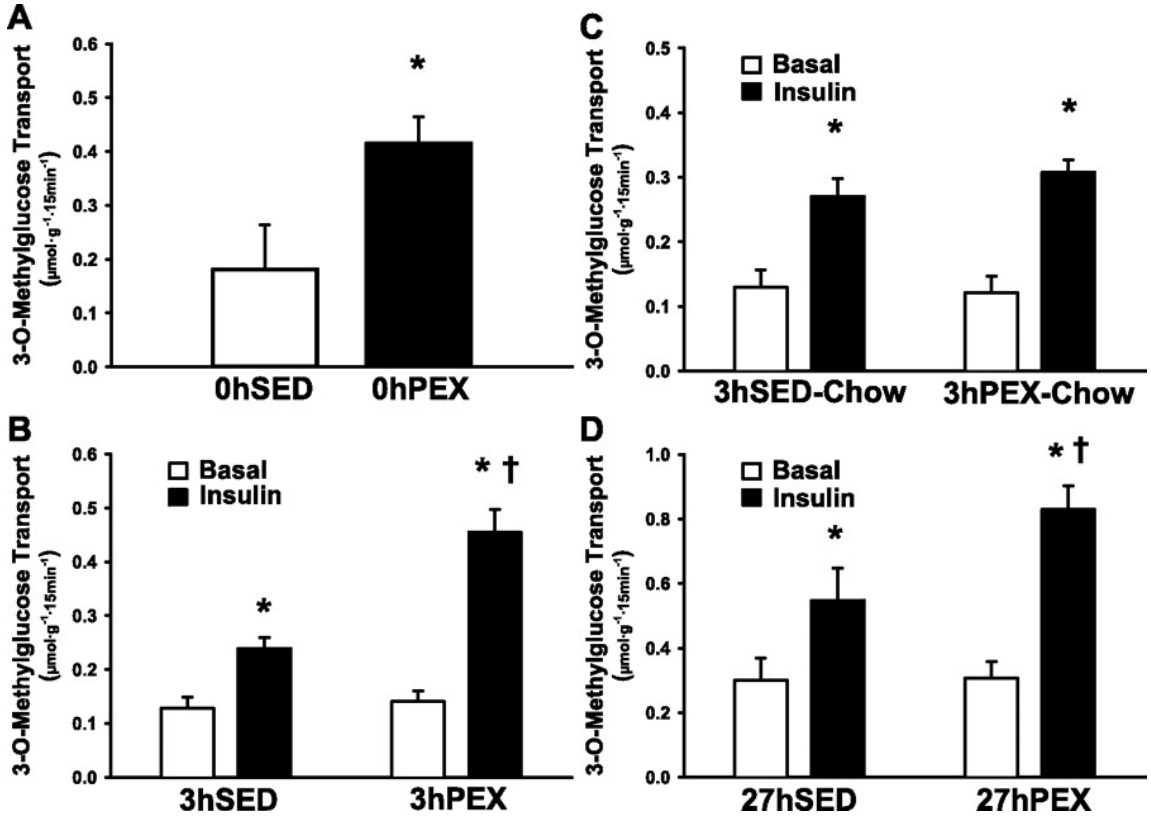

Figure 5.1
Rate of 3-O-methylglucose (3-MG) transport in isolated rat epitrochlearis muscles. A) 0hPEX, B) 3hPEX, C) 3hPEX-Chow, and D) 27hPEX. For rats in the 0hPEX and 0hSED groups, all muscles that were used to measure 3-MG transport were incubated without insulin to determine the insulin-independent effect of exercise. For rats in the 3hPEX, 3hPEX-Chow and 27hPEX groups and their respective SED controls, one of the paired muscles was incubated without insulin and the contralateral muscle was incubated with insulin. A) Data are means ± SE, n = 6 per group. *P < 0.05 (exercise effect; t-test). Open bar = 0hSED; grey bar = 0hPEX. B-D) Data are means ± SE, n = 6-13 per 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.

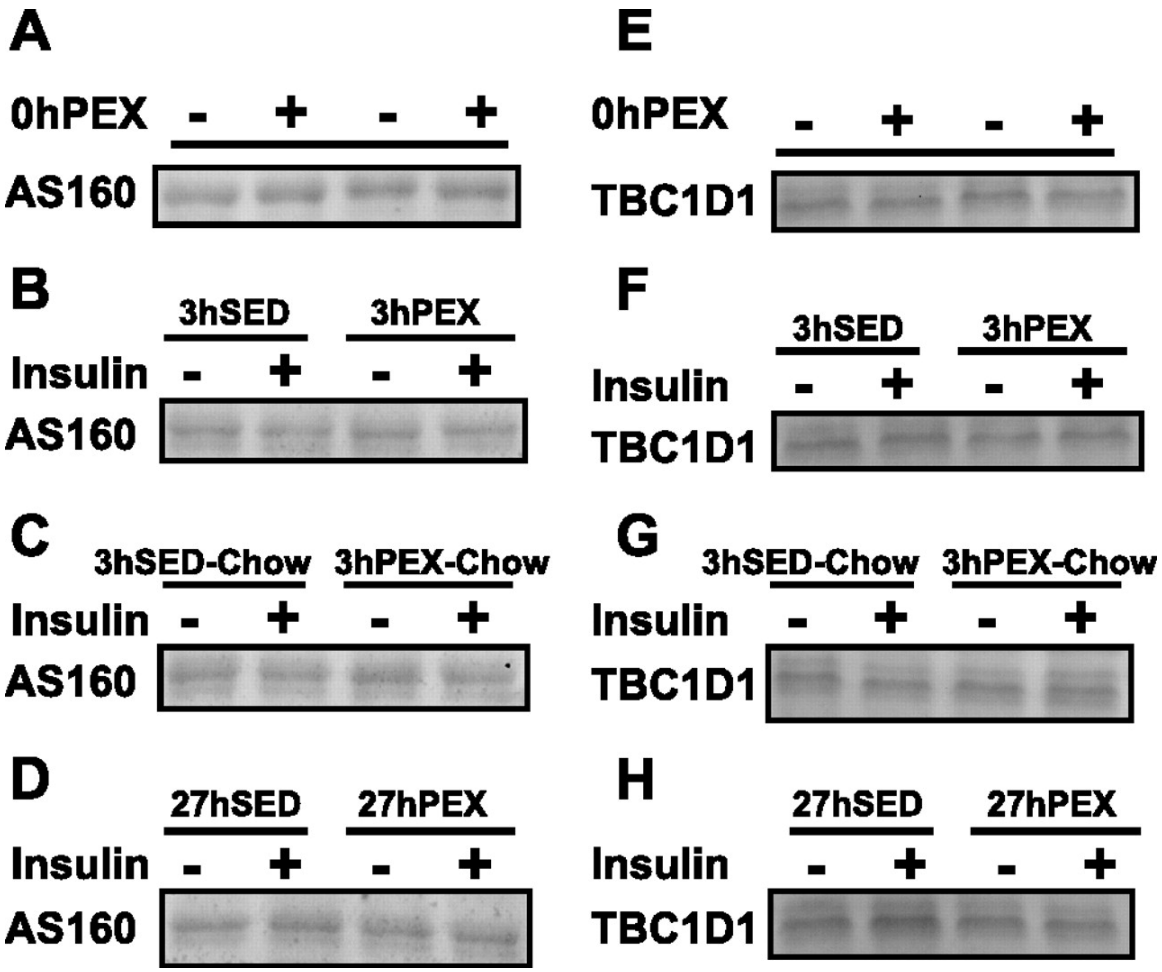

Figure. 5.2
Akt substrate of 160 kDa (AS160) and TBC1D1 total protein abundance in rat epitrochlearis muscles. There were no significant effects of exercise or insulin with any of the protocols (n = 4 for each exercise and insulin treatment group). *A*: AS160 of 0-h PEX; *B*: AS160 of 3-h PEX; *C*: AS160 of 3-h PEX-chow; *D*: AS160 of 27-h PEX; *E*: TBC1D1 of 0-h PEX; *F*: TBC1D1 of 3-h PEX; *G*: TBC1D1 of 3-h PEX-chow; *H*: TBC1D1 of 27-h PEX.

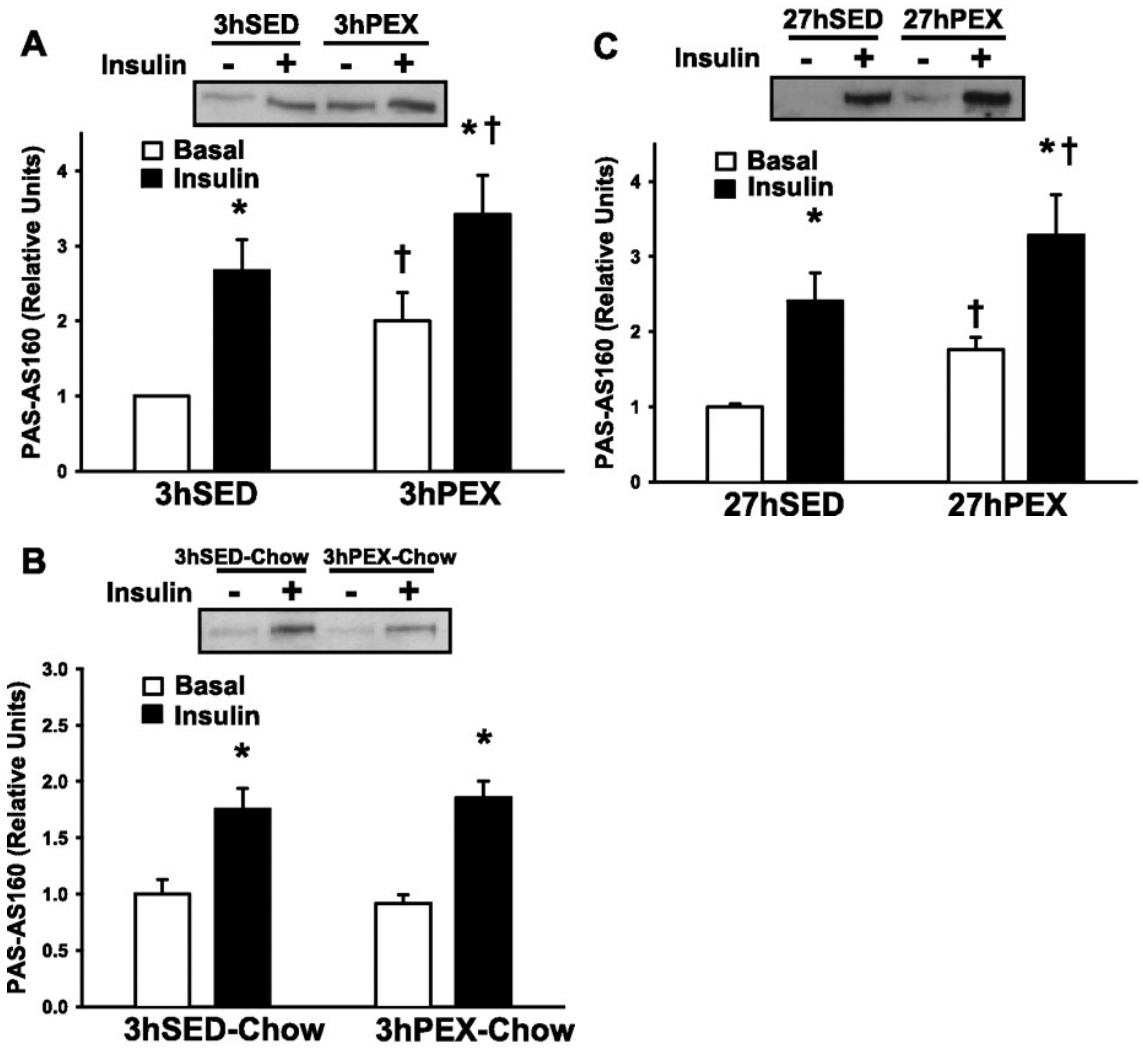

Figure 5.3

AS160 PAS-phosphorylation in rat epitrochlearis muscles. A) 3hPEX, B) 3hPEX-Chow, and C) 27hPEX. One of the paired muscles from each rat was incubated without insulin and the contralateral muscle was incubated with insulin. Data are means ± SE, n = 6-13 per 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. The SE value for the basal 3hSED group Fig. 3A is too small to be visible.

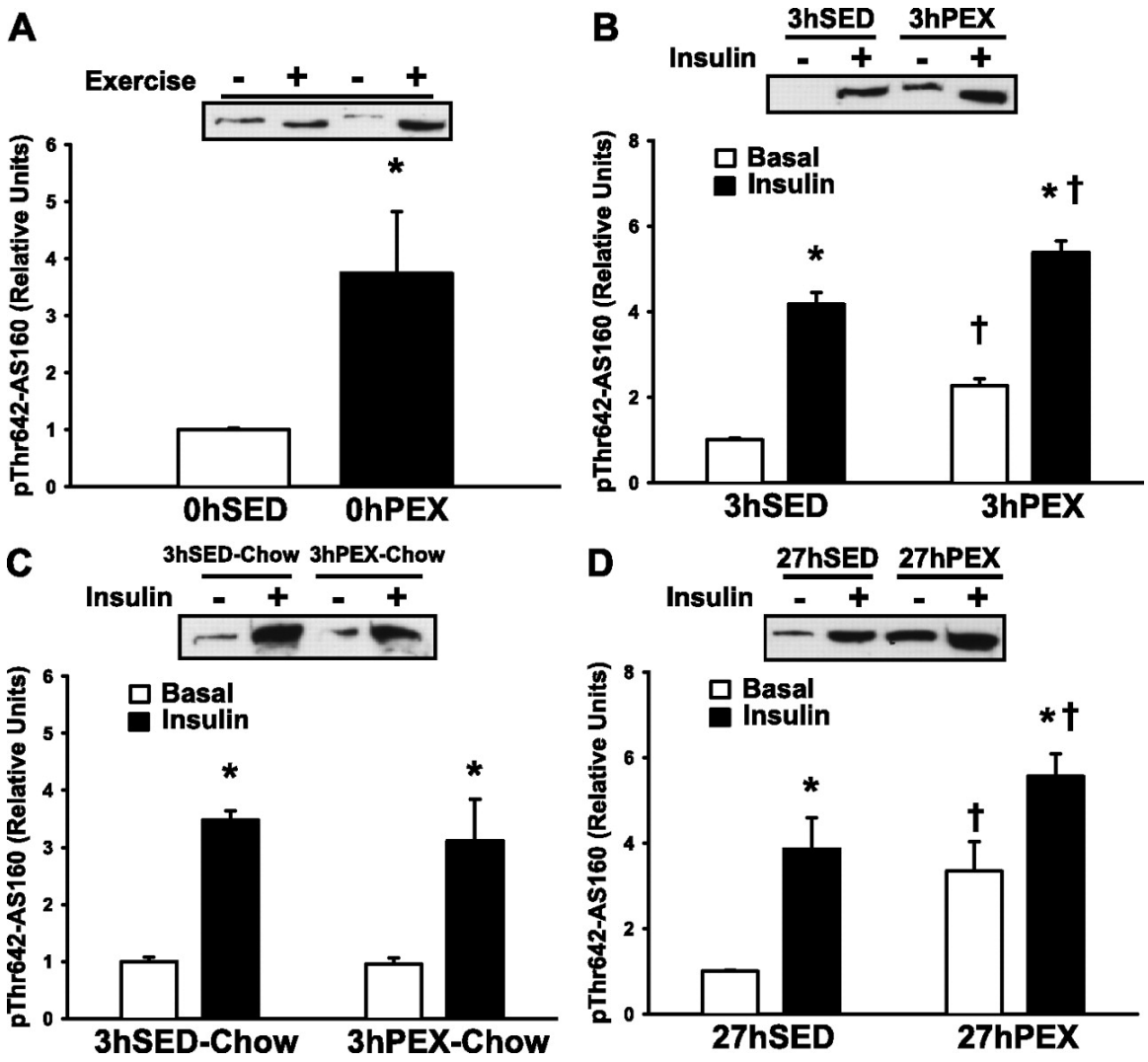

Figure 5.4
AS160 Thr642 phosphorylation in rat epitrochlearis muscles. A) 0hPEX, B) 3hPEX, C) 3hPEX-Chow, and D) 27hPEX. For rats in the 0hPEX group, muscles were frozen immediately after dissection and used to determine the insulin-independent effect of exercise. For rats in the 3hPEX, 3hPEX-Chow and 27hPEX groups and their respective SED controls, one paired muscle from each rat was incubated without insulin and the contralateral muscle was incubated with insulin. A) Data are means ± SE, n = 4 per group. *P < 0.05 (exercise effect; t-test). Open bar = 0hSED; grey bar = 0hPEX. B-D) Data are means ± SE, n = 4 per 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.

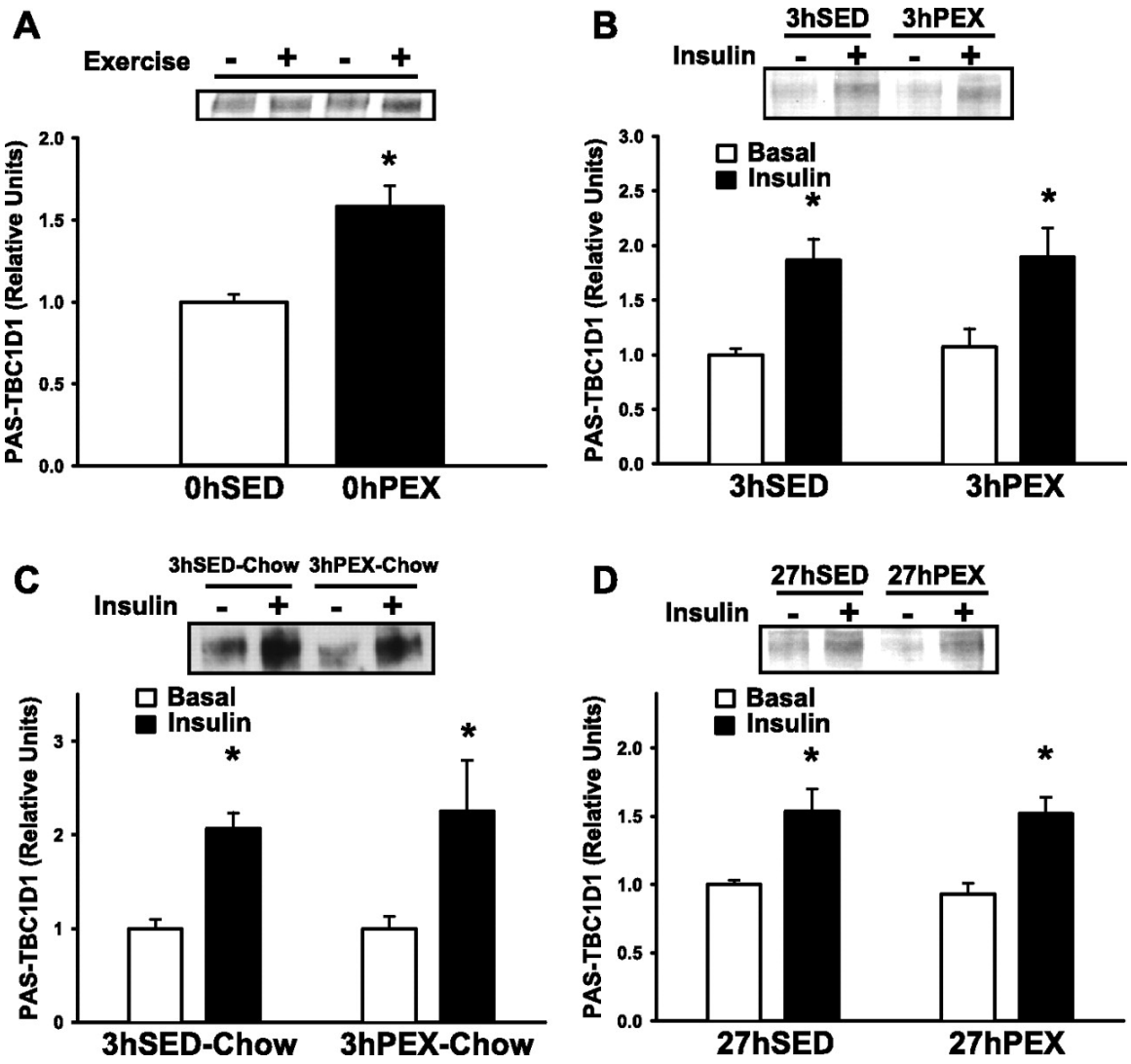

Figure 5.5

TBC1D1 PAS-phosphorylation in rat epitrochlearis muscles. A) 0hPEX, B) 3hPEX, C) 3hPEX-Chow, and D) 27hPEX. For rats in the 0hPEX group, muscles were frozen immediately after dissection and used to determine the insulin-independent effect of exercise. For rats in the 3hPEX, 3hPEX-Chow and 27hPEX groups and their respective SED controls, one of the paired muscles from each rat was incubated without insulin and the contralateral muscle was incubated with insulin. A) Data are means ± SE, n = 6 per group. *P < 0.05 (exercise effect; t-test). Open bar = 0hSED; grey bar = 0hPEX. B-D) Data are means ± SE, n = 6-10 per group. *P < 0.05 (insulin effect; post-hoc test). Open bars = without insulin; filled bars = with insulin.

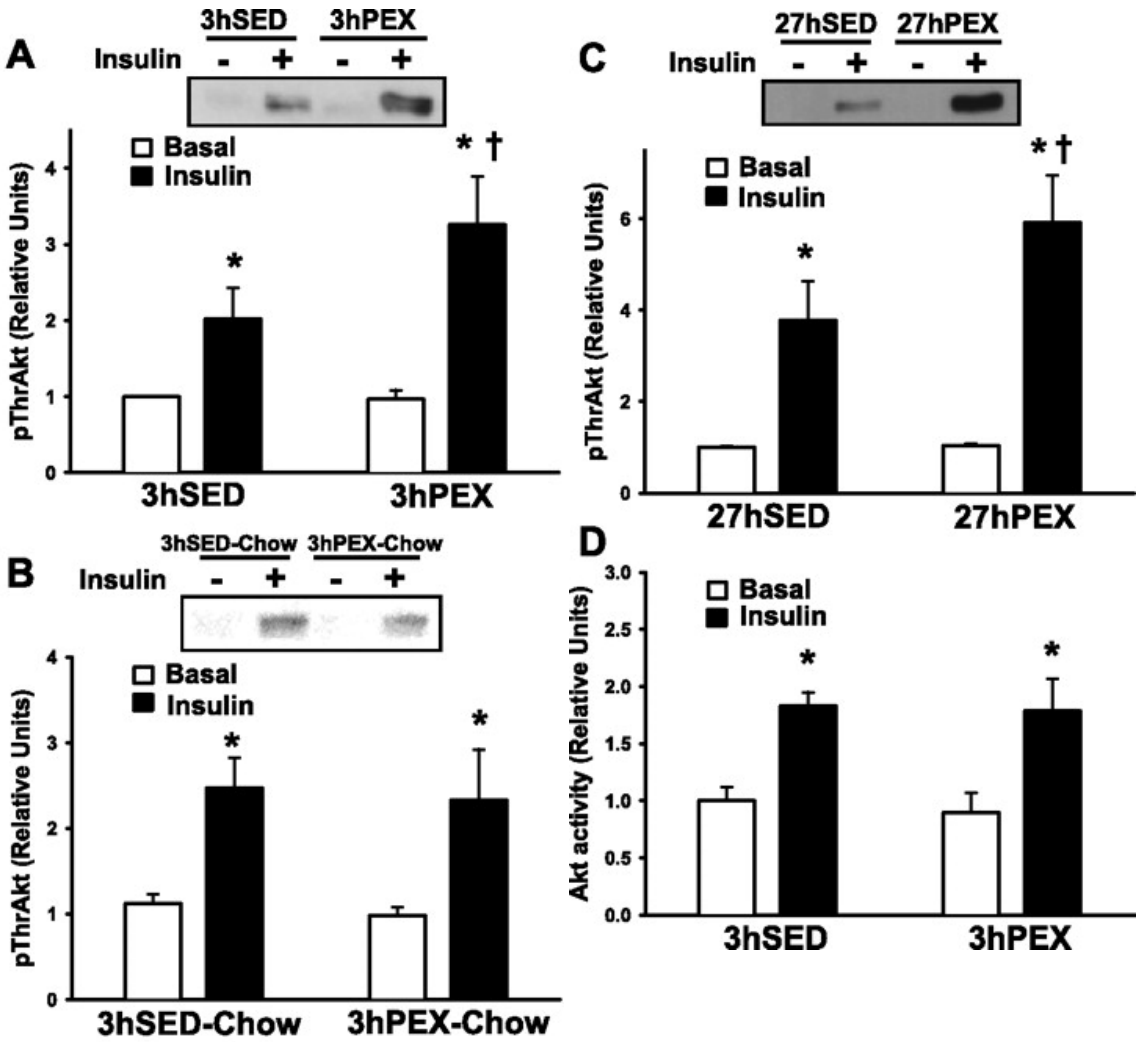

Figure 5.6

Akt threonine phosphorylation and Akt activity in rat epitrochlearis muscles. One of the paired muscles from each rat was incubated without insulin and the contralateral muscle was incubated with insulin. A) pThrAkt 3hPEX, B) pThrAkt 3hPEX-Chow, C) pThrAkt 27hPEX, and D) Akt activity 3hPEX. Data are means ± SE, n = 7-12 per 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. Open bars = without insulin; filled bars = with insulin. The SE value for the basal 3hSED group in Fig. 6A is too small to be visible.

Time, h	SED	PEX	SED-Chow	PEX-Chow
0	14.4±1.5*	5.9±0.6#		
3	13.5±1.9*	5.8±1.1#	17.5±2.2*	25.9±1.8†
27	9.4±0.3#	8.8±1.1#		

Table 5.1
Muscle glycogen concentration. Values are means ± SE (μmol/g muscle); *n* = 6-8/group. SED, sedentary; PEX, postexercise; SED- and PEX-chow, SED and PEX rats, respectively, with unlimited access to food. Time refers to the time at which muscles were sampled relative to the completion of exercise by PEX groups. Values not marked with the same symbol (*, #, or †) were significantly different ($P < 0.05$). 0 = immediately PEX; 3 = 3 h PEX; 27 = 27 h PEX.

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CHAPTER VI

STUDY 4

In Vivo Exercise Followed by In Vitro Contraction Additively Elevates Subsequent Insulin-stimulated Glucose Transport by Rat Skeletal Muscle

ABSTRACT

The cellular mechanisms whereby prior exercise enhances insulin-stimulated glucose transport are not well understood. Previous studies suggested that a prolonged increase in phosphorylation of Akt substrate of 160 kDa (AS160), a Rab GTPase-activating protein (RabGAP) that is implicated in the regulation of glucose transport, may be important for the post-exercise increase in insulin sensitivity. Isolated skeletal muscles that are stimulated to contract in vitro only have a subsequent increase in insulin-stimulated glucose transport when incubated in serum during contraction. In this study, dissected rat epitrochlearis muscles were stimulated to contract with or without serum to identify whether increased insulin-dependent glucose transport coincides with enhanced AS160 or TBC1D1 (a paralog of AS160) phosphorylation at 3 h post-electrical stimulation. An increase in insulin-dependent glucose transport occurred in the absence of enhanced AS160 or TBC1D1 phosphorylation 3 h after electrical stimulation, suggesting that increased insulin sensitivity in isolated skeletal muscle that were stimulated to contract occur independent of increased phosphorylation of these two

RabGAP proteins. Importantly, the combined effects of prior exercise and prior electrical stimulation in the same muscles on a subsequent increase in insulin-stimulated glucose transport were additive, suggesting that exercise and electrical stimulation may stimulate insulin-stimulated glucose transport through distinct mechanisms. It remains possible that in vivo exercise stimulated the subsequent increase in insulin sensitivity through increased AS160 phosphorylation, whereas an unidentified mechanism may be important for the electrical stimulation effect on insulin sensitivity.

INTRODUCTION

A single bout of exercise leads to: 1) an increase in insulin-independent glucose transport during and immediately after exercise, and 2) a subsequent increase in insulin-dependent glucose transport (5, 24). The enhanced insulin-stimulated glucose transport post-exercise occurs as a result of greater insulin-stimulated cell-surface GLUT4 localization (16), but the cellular mechanisms that lead to this event are not well understood (5). Many studies have found that prior exercise does not amplify insulin effects on proximal insulin signaling steps (e.g., insulin receptor tyrosine kinase activity, insulin receptor substrate tyrosine phosphorylation, insulin receptor substrate association phosphatidylinositol-3 kinase activity, Akt serine phosphorylation, etc.) (1, 8, 15, 29, 30). In contrast, a number of studies have recently found a prolonged increase in phosphorylation of Akt substrate of 160 kD (AS160 also known as TBC1D4) after exercise (1, 12, 26, 28). We previously proposed that this persistent increase in AS160 phosphorylation may be important for the post-exercise enhancement of insulin-stimulated glucose transport (1, 3, 12).

AS160 and TBC1D1 are paralog Rab GTPase-activating proteins (RabGAP) that appear to regulate GLUT4 vesicular trafficking (6, 17, 18, 23, 25). In basal conditions, AS160 appears to restrain GLUT4 exocytosis presumably by its active RabGAP domain. Insulin-stimulated phosphorylation of AS160 on specific motifs appears to inactivate its RabGAP domain, thus allowing GLUT4 to become translocated (19, 25). In contrast, evidence from cultured cells suggests that insulin may be less effective in regulating TBC1D1's restraint of GLUT4 translocation (6, 7, 17, 23). Results from skeletal muscle are consistent with the idea that phosphorylation of TBC1D1 may be important for an increase in insulin-independent glucose transport immediately after exercise (11, 12, 22). Nevertheless, enhanced phosphorylation of TBC1D1 had reversed at 3 h post-exercise, suggesting it may not be essential for the persistently increased insulin sensitivity post-exercise (12).

Although in vivo exercise results in a subsequent increase in insulin-stimulated glucose transport, isolated skeletal muscle that are electrically stimulated to contract in serum-free buffer do not exhibit enhanced insulin-stimulated glucose transport (4). Gao et al. found that when isolated skeletal muscles were stimulated to contract in serum, there was a subsequent increase in insulin-stimulated glucose transport (13). Therefore, they hypothesized that a serum factor may be essential for the post-exercise increase in insulin-stimulated glucose transport (13).

To further investigate the role that increased phosphorylation of AS160 may play on insulin sensitivity, isolated skeletal muscle was stimulated to contract with or without serum. Glucose transport and phosphorylation of AS160 and TBC1D1 were measured immediately or 3 h after electrical stimulation. We hypothesized that in vitro contraction

results in 1) increased insulin-independent glucose transport and AS160 phosphorylation immediately after electrical stimulation regardless of the presence of serum, but 2) serum would be required for a persistent increase in AS160 phosphorylation and enhanced insulin-stimulated glucose transport 3 h after electrical stimulation and 3) the effects of exercise and electrical stimulation would not be additive for insulin-stimulated glucose transport or AS160 phosphorylation, consistent with the idea that they share the same mechanism (increased AS160 phosphorylation) for increased insulin sensitivity.

METHODS

Materials. Serum from male Wistar rats (120-200g, fasted for 12 hr) was purchased from Gemini Bio-Products (West Sacramento, CA). 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 from Pierce Biotechnology (Rockford, IL). Anti-phospho-Thr^{308}Akt (pThr^{308}Akt, no. 9275), anti-phospho-Thr^{172}AMPK (pThr^{172}AMPK, no. 2531), anti-phospho-(Ser/Thr) Akt substrate (PAS, no. 9611), and goat anti-rabbit IgG HRP conjugate (no. 7074) were from Cell Signaling Technology (Danvers, MA). PAS was designed to recognize Akt phosphorylation motif peptide sequences (RXRXXpT/S). Total TBC1D1 and phospho-Ser^{237} (equivalent to Ser^{237} in humans and Ser^{231} in rats) TBC1D1 (pSer^{237}TBC1D1) polyclonal antibody and were provided by Dr. Makoto Kanzaki at Tohoku University (20). Anti-AS160 (no. 07-741), anti-phospho-Thr^{642} (equivalent to Thr^{642} in humans and

Thr^{597} in rats) AS160 (pThr^{642}AS160, no. 07-802) and protein G agarose beads (no. 16-266) were from Upstate USA (Charlottesville, VA). 3-*O*-Methyl-[^{3}H]glucose ([^{3}H]3-MG) was from Sigma-Aldrich. [^{14}C]Mannitol was from Perkin Elmer (Waltham, MA). Other reagents were from Sigma-Aldrich and Fisher Scientific (Pittsburgh, PA).

Insulin concentration in serum. Rat serum purchased from Gemini Bio-Products was submitted to the Chemistry Laboratory Core of the Michigan Diabetes Research and Training Center for the measurement of insulin concentration. The insulin concentration of serum (21 μU/mL) was determined with a double-antibody radioimmunoassay using an ^{125}I-Human insulin tracer (Linco Research), a rat insulin standard (Novo), a guinea pig anti-rat insulin first antibody (Linco Research), and a sheep anti-guinea pig gamma globulin-PEG second antibody (MDRTC).

Animal treatment. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. Male Wistar rats (120–200 g; Harlan, Indianapolis, IN) were provided with rodent chow (Lab Diet; PMI Nutritional International, Brentwood, MO) and water ad libitum until 1700 the night before the experiment when their food was removed. On the following day between 800 and 1000, rats were randomly assigned to: 1) resting (REST), 2) post-electrical stimulation (in vitro electrical stimulation: PES), 3) post-exercise (in vivo exercise: PEX) or 4) post-exercise and electrical stimulation (in vivo exercise followed by in vitro electrical stimulation: PEX+PES) groups. For all experiments, rats were anesthetized with an intrapertoneal injection of sodium pentobarbital (50 mg/kg wt). While rats were under deep anesthesia, both epitrochlearis muscles were rapidly dissected.

In vitro electrical stimulation. Paired isolated epitrochlearis muscles were incubated in either Krebs-Henseleit buffer (KHB) + 0.1% bovine serum albumin (BSA) + 8 mM glucose + 32 mM mannitol (Solution 1) or in serum for 30 min in a water bath at 35°C. For all incubation steps, flasks were continuously gassed from above with 95% O_2/5% CO_2 and shaken in a heated water bath. Subsequently, one of the paired muscles was transferred to vials containing identical media (REST). The contralateral muscle was attached to a glass rod and a force transducer (Radnoti, Litchfield, CT). Mounted muscles were incubated in identical media and were stimulated to contract (PES) as previously described (Grass S48 Stimulator, Grass Instruments, Quincy, MA; 0.1 ms twitch, 100 Hz train for 10 s, 10 trains, 10 min) (9). For the experiments immediately post-electrical stimulation (0hREST and 0hPES, Fig. 6.1A), muscles were either freeze-clamped or transferred to vials containing KHB + 0.1% BSA + 2 mM pyruvate + 36 mM mannitol (Solution 2; 30°C, 10 min) prior to 3-MG transport measurement. For the experiments 3 h post-electrical stimulation (3hREST and 3hPES, Fig. 6.2A and 6.S2A), muscles were incubated according to the protocol previously used (9, 13). Immediately after electrical stimulation, all muscles (regardless of whether the previous incubation was with or without serum) were transferred to vials containing Solution 1 for a 5 min wash step at 35°C. Muscles were then transferred to other vials containing Solution 1 for 3 h at 35°C. After 3 h, muscles were transferred to flasks containing Solution 2 for 30 min at 30°C. During this step, for some muscle pairs, Solution 2 contained 50 μU/mL of insulin; for other muscle pairs Solution 2 contained no insulin. After 30 min, all muscles were incubated with 3-MG (see 3-MG transport below).

Serum vs. insulin experiment. One experiment compared the effect of incubation of muscles in serum to incubation of muscles in 21 μU/mL insulin (which equaled the insulin concentration in serum; Fig. 6.S1A). Immediately after dissection, isolated epitrochlearis muscles were placed in Solution 1 with no insulin, Solution 1 with 21 μU/mL of insulin, or serum for 30 min at 35°C. Muscles were then transferred to a second flask including the identical media as in Step 1 for 10 min before being freeze-clamped.

Additivity experiments. In experiments comparing the combined effects of exercise and electrical stimulation on the subsequent increase in insulin-stimulated glucose transport, four groups were studied: REST, PES, PEX and PEX+PES. Prior to anesthetization and muscle incubation steps, PEX or PEX+PES rats swam in a barrel filled with water (35°C) to a depth of ~60 cm (6-8 rats/barrel) for 4 x 30 min bouts, with a 5-min rest period between each bout. Immediately after exercise or rest, both epitrochlearis muscles were rapidly removed and were either frozen immediately (0hPEX or 0hREST, Fig. 6.4A) or were frozen after subsequent in vitro electrical stimulation (0hPEX+PES or 0hPES, Fig 6.4A). In another set of experiments, immediately after exercise or rest, both epitrochlearis muscles were dissected out and incubated in serum prior to and during electrical stimulation (or resting), followed by a 5 min wash in Solution 1, a 3 h rest in Solution 1 and a 30 min rinse in Solution 2 (± insulin) prior to incubation with 3-MG as described below (3hREST, 3hPES, 3hPEX and 3hPEX+PES, Fig. 6.3A).

3-MG transport and homogenization. After incubation in Solution 2, muscles were transferred to flasks containing KHB + 0.1% BSA + 8 mM 3-MG (including [^{3}H]3-

MG at a final specific activity of 0.25 mCi/mmol), and 2 mM mannitol (including [^{14}C]mannitol at a final specific activity of 6.25 μCi/mmol) with or without 50 μU/mL insulin at 30°C. After 10 min, muscles were rapidly blotted, trimmed, freeze-clamped and stored (-80°C) until processed.

Frozen muscles were homogenized in 1 ml ice-cold homogenization buffer (2 mM Na_3VO_4, 2 mM EDTA, 2 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM ß-glycerophosphate, 1 mM phenylmethanesulphonylfluoride, and 1 μg/ml leupeptin in T-PER) using glass-on-glass tubes (Kontes, Vineland, NJ). Homogenates were subsequently rotated at 4°C for 1 h before being centrifuged (15,000 *g* for 10 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 was determined as previously described (2). A portion of supernatant was used to determine protein concentration according to the manufacturer's instructions (Pierce Biotechnology Catalog no. 23227). The remaining supernatant was stored at –80°C until further analyzed.

Immunoprecipitation. Homogenized muscle (200-300 μg protein) was precleared and immunoprecipitated with anti-AS160 or anti-TBC1D1 at 4°C with protein G agarose beads. After gentle rotation overnight, the immunoprecipitation mix was centrifuged at 4,000 *g*, and the supernatant was aspirated. After washing (four times with 500 μl phosphate-buffered saline), the protein bound to the beads was eluted with 2x SDS loading buffer and boiled before loading on a polyacrylamide gel.

Immunoblotting. Samples (immunoprecipitates or homogenates) were boiled with SDS loading buffer, separated by PAGE, and electrophoretically transferred to

nitrocellulose. Samples 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% nonfat dry milk in TBST for 1 h at room temperature, washed 3 x 5 min at room temperature, and treated with the primary antibodies (1:1,000 in TBST + 5% BSA) overnight at 4°C. Blots were then washed 3 x 5 min with TBST, incubated with the secondary antibody, goat anti-rabbit IgG HRP conjugate (1:20,000 in TBST + 5% milk), for 1 h at room temperature, washed again 3 x 5 min with TBST, and developed with West Dura Extended Duration Substrate reagent. Protein bands were quantified by densitometry (Alpha Innotech, San Leandro, CA). The mean values for basal muscles (REST without insulin) on each blot were normalized to equal 1.0, and then all samples on the blot were expressed relative to the normalized basal value.

Muscle glycogen concentration. Muscles used for measurement of glycogen were weighed and then homogenized in ice-cold 0.3 M perchloric acid. An aliquot of the homogenate was stored at -80ºC for later determination of glycogen concentration by the amyloglucosidase method (21).

Statistical analysis. Statistical analyses were performed using Sigma Stat version 2.0 (San Rafael, CA). Data were expressed as mean ±SE. $P \leq 0.05$ was considered statistically significant. Data from the REST vs. PES (0h and 3h) experiments were analyzed with two-way ANOVA. Data from the serum vs. insulin experiment were analyzed using one-way ANOVA. Data from the additivity experiments were analyzed with: 1) one-way ANOVA for those muscles that were frozen immediately after exercise and/or electrical stimulation (0hPEX+PES) or 2) two-way ANOVA for those muscles that were frozen 3 h after exercise and/or electrical stimulation (3hPEX+PES). For all

ANOVA, the Student-Newman-Keuls post-hoc test was used to identify the source of significant variance. When data failed the Levene Median test for equal variance, the Kruskal-Wallis nonparametric ANOVA on ranks was used with Dunn's post-hoc test.

RESULTS

Immediately post-electrical stimulation. Glucose transport determined immediately after the electrical stimulation (0hPES) was significantly greater ($P < 0.05$) compared with 0hREST values, regardless of the incubation media (serum-free buffer or serum) (Fig. 6.1B). Electrical stimulation (0hPES) also resulted in significantly greater pThr^{308}Akt, pThr^{172}AMPK, PAS-AS160, pThr^{642}AS160, PAS-TBC1D1, and pSer^{237}TBC1D1 compared with the 0hREST group, regardless of the incubation media (serum-free buffer or serum) ($P < 0.05$, Fig. 6.1C-H).

In the muscles that were incubated in serum prior to and during electrical stimulation (or resting), glucose transport (measured immediately after ± electrical stimulation) was significantly elevated compared to the muscles that were incubated in serum-free buffer prior to and during the electrical stimulation step (Fig. 6.1B). Incubation in serum had no effect on pThr^{172}AMPK and pSer^{237}TBC1D1 (Fig. 6.1D, H). In contrast, muscles that were incubated in serum prior to and during electrical stimulation (or resting) had greater values for pThr^{308}Akt, PAS-AS160 and pThr^{642}AS160 ($P < 0.05$) compared to the muscles that were incubated in serum-free buffer prior to and during electrical stimulation step (Fig. 6.1C, E, F). Incubation in serum resulted in greater PAS-TBC1D1 (compared to incubation in serum-free buffer) in muscles that were not stimulated to contract ($P < 0.05$), but incubation in serum had no effect on PAS-

TBC1D1 (compared to incubation in serum-free buffer) in muscles that were stimulated to contract (Fig. 6.1G). Incubating muscles in insulin at concentration equivalent to that in the serum (21 μU/mL) resulted in levels of pThr^{308}Akt, PAS-AS160, pThr^{642}AS160 and PAS-TBC1D1 that were greater compared to muscles incubated in serum-free buffer ($P < 0.05$) but not different from muscles incubated in serum (Fig. 6.S1). Thus, the effects of serum alone on these outcomes could be accounted for by the effects produced by the insulin concentration that was found in the serum.

3 h post-electrical stimulation without serum. As previously reported (4, 9, 13), prior electrical stimulation in serum-free buffer (3hPES) had no effect on glucose transport in basal or insulin-stimulated muscles (measured 3 h after electrical stimulation) compared to 3hREST (Fig. 6.S2B). As expected, prior electrical stimulation had no effect on the phosphorylation of any of the proteins studied (pThr^{308}Akt, pThr^{172}AMPK, PAS-AS160, pThr^{642}AS160, PAS-TBC1D1, and pSer^{237}TBC1D1) in basal or insulin-stimulated muscles at 3hPES compared to 3hREST (Fig. 6.S2C-H). Insulin treatment (immediately prior to and during the incubation with 3-MG) significantly elevated 3-MG transport, pThr^{308}Akt, PAS-AS160, pThr^{642}AS160 and PAS-TBC1D1 ($P < 0.05$, Fig. 6.S2B-C, E-G) compared to no insulin treatment, but had no effect on pThr^{172}AMPK or pSer^{237}TBC1D1 (Fig. 6.S2D, H).

3 h post-electrical stimulation with serum. As previously reported (9, 13), prior electrical stimulation in the presence of serum (3hPES) resulted in greater insulin-stimulated glucose transport (measured 3 h after electrical stimulation) compared to 3hREST ($P < 0.05$, Fig. 6.2B). In contrast, prior electrical stimulation had no effect on the phosphorylation of any of the proteins studied (pThr^{308}Akt, pThr^{172}AMPK, PAS-

AS160, pThr^{642}AS160, PAS-TBC1D1, and pSer^{237}TBC1D1) in basal or insulin-stimulated muscles at 3hPES compared to 3hREST (Fig. 6.2C-H). Insulin treatment (immediately prior to and during the incubation with 3-MG) significantly elevated 3-MG transport, pThr^{308}Akt, PAS-AS160, pThr^{642}AS160 and PAS-TBC1D1 ($P < 0.05$, Fig. 6.2B-C, E-G) compared to no insulin treatment, but had no effect on pThr^{172}AMPK or pSer^{237}TBC1D1 (Fig. 6.2D, H).

Additivity experiments. The purpose of these experiments was to examine the possibility that prior in vitro contraction (in serum) enhances insulin-stimulated glucose transport through a mechanism distinct from that after exercise. Insulin-stimulated glucose transport in muscles from rats that exercised immediately prior to electrical stimulation of the isolated muscles (PEX+PES) was compared to that of muscles that were subjected to PEX or PES treatment alone (Fig. 6.3B). Total force production during electrical stimulation (11,102.3 ± 755.5 g·min·g muscle^{-1} vs. 8969.8 ± 1129.2 g·min·g muscle^{-1}, $P < 0.05$), but not peak force (478.0 ± 26.1 g·g muscle^{-1} vs. 424.5 ± 52.4 g·g muscle^{-1}, $P = 0.122$), was significantly reduced in PEX+PES muscles compared to PES muscles. Either exercise (3hPEX, Fig. 6.3C, 3rd bar) or electrical stimulation (3hPES, Fig. 6.3C, 2nd bar) induced a subsequent increase in insulin-stimulated glucose transport, with no significant differences between the groups. Importantly, muscles that underwent electrical stimulation after being dissected from exercised rats (3hPEX+PES, Fig. 6.3C, 4th bar) had greater insulin-stimulated glucose transport compared to muscles from the 3hPEX or 3hPES group. The greater insulin-stimulated glucose transport in 3hPEX+PES muscles was not accompanied by altered pThr^{642}AS160, PAS-TBC1D1 or GLUT4 abundance in the 3hPEX+PES group versus the PEX group (Fig. 6.3D-F). Furthermore,

glycogen concentration, pThr^{172}AMPK and pThr^{642}AS160 were not different for the 0hPEX+PES group compared to the 0hPES group (Fig. 6.4B-D). Doubling the amount of exercise (1 h vs. 2 h) or electrical stimulation (5 tetani vs. 10 tetani) did not result in higher values for the subsequent insulin-stimulated glucose transport and pThr^{642}AS160 (Fig. 6.5 and 6.6), providing evidence that the protocol used for each stimulus (exercise or electrical stimulation) was maximally effective.

DISCUSSION

A primary aim of this study was to determine if the enhanced insulin-stimulated glucose transport 3 h after in vitro electrical stimulation of isolated skeletal muscle in serum would be accompanied by elevated AS160 phosphorylation as occurs after in vivo exercise. Consistent with previous reports (4, 9, 10, 13, 14), in vitro electrical stimulation of isolated rat epitrochlearis muscles resulted in: 1) a subsequent increase in insulin sensitivity 3 h after electrical stimulation when incubated in rat serum; and 2) unaltered insulin sensitivity 3 h after electrical stimulation in serum-free buffer. However, neither group was characterized by a prolonged increased AS160 phosphorylation at 3 h post-contraction despite an elevated AS160 phosphorylation immediately after in vitro contraction. These results contrast with the prolonged increase in AS160 phosphorylation that accompanied the enhanced insulin-stimulated glucose transport at 3 and 27 h after in vivo exercise (1, 12). Importantly, insulin-stimulated glucose transport in muscles from rats that had undergone in vivo exercise immediately prior to in vitro electrical stimulation (PEX+PES) was greater than values found in muscles after either in vivo exercise (PEX) or in vitro electrical stimulation (PES) alone. These results provide

evidence to suggest that in vivo exercise and in vitro electrical stimulation may stimulate enhanced insulin-stimulated glucose transport through distinct mechanisms. The essentially additive effects of in vivo exercise and in vitro contraction on the insulin-stimulated glucose transport in the PEX+PES group was not attributable to differences in GLUT4 abundance, glycogen depletion or phosphorylation of AS160, TBC1D1 or AMPK.

Arias et al. (1) found a prolonged increase in AS160 phosphorylation concomitant with improved insulin-stimulated glucose transport 3-4 h after in vivo exercise and suggested that the enhanced AS160 phosphorylation may play a role in post-exercise increase in insulin-stimulated glucose transport. Consistent with this idea, we recently extended this observation by demonstrating that: 1) at 27 h post-exercise both AS160 phosphorylation and insulin-stimulated glucose transport remained elevated in fasted rats, and 2) when rats were allowed to eat a high-carbohydrate chow for 3 h after exercise, the increased AS160 phosphorylation and the increased insulin-stimulated glucose transport were both reversed to levels found in sedentary controls (12). Other studies have also supported that AS160 phosphorylation remains enhanced several hours post-exercise in humans (26, 28). In contrast, in vitro contraction in serum-free buffer has not been found to induce a subsequent increase in insulin-stimulated glucose transport (4, 13), but contraction in serum does lead to increased insulin sensitivity (9, 13). Hence, we hypothesized that a protocol for in vitro electrical stimulation in serum that was known to result in a subsequent increase in insulin-stimulated glucose transport would also induce a prolonged increase in AS160 phosphorylation. As expected, insulin-stimulated glucose transport was enhanced 3 h after in vitro contraction in serum. However, it did not result

in a prolonged increase in AS160 phosphorylation (measured by PAS-AS160 and pThr^{642}AS160) at 3hPES. Therefore, enhanced insulin-stimulated glucose transport in 3hPES group appears to occur independent of enhanced AS160 phosphorylation, at least at Thr^{642}. Treebak et al., recently reported that prior exercise resulted in enhanced phosphorylation of AS160 on Ser^{318}, Ser^{341} and Ser^{751} (28). It remains possible that phosphorylation on these sites are enhanced 3 h after in vitro electrical stimulation.

TBC1D1, a paralog protein of AS160 (or TBC1D4), has also been implicated in the regulation of GLUT4 translocation (6, 11, 23, 27). Recently we demonstrated that PAS-TBC1D1 was not enhanced 3 h after in vivo exercise (12). It remained possible that phosphorylation of TBC1D1 might play a role in enhanced insulin sensitivity with in vitro electrical stimulation in the presence of serum. However, TBC1D1 phosphorylation (measured by PAS-TBC1D1 or pSer^{237}TBC1D1) was unchanged for 3hPES vs. 3hREST values, suggesting that enhanced insulin sensitivity after electrically stimulated contraction in vitro occurs independent of enhanced phosphorylation of TBC1D1 Ser^{237}.

At first glance, the results with in vitro contraction may be interpreted to suggest that enhanced AS160 phosphorylation post-exercise is only incidental to the enhanced insulin-stimulated glucose transport after in vivo exercise. However, this interpretation is based on the assumption that exercise and electrical stimulation enhance insulin-stimulated glucose transport through an identical mechanism. Although that is one possibility, there are two other possibilities. The mechanisms for elevated insulin-stimulated glucose transport PEX and PES may be: 1) mutually exclusive (in which event, it remains possible that AS160 phosphorylation is important for elevated insulin sensitivity PEX, but not PES); or 2) partially overlapping (in which event AS160

phosphorylation may or may not be important for a part of the PEX improvement in insulin sensitivity). In an attempt to distinguish among these possibilities, muscles from rats that had undergone in vivo exercise prior to in vitro electrical stimulation (PEX+PES) were compared to muscles after either in vivo exercise (PEX) or in vitro electrical stimulation (PES) alone. Importantly, insulin-stimulated increase in glucose transport (Δ-insulin, calculated by subtracting glucose transport in muscles incubated without insulin from glucose transport in muscles incubated with insulin) in PEX+PES group was significantly greater than that of PEX or PES group. Furthermore, the effect was essentially additive as evidenced by the approximately equivalent values for sum of 1) Δ-insulin in 3hPEX group above that of 3hREST group (0.156 μmol/g/10min) and 2) Δ-insulin in 3hPES group above that of 3hREST group (0.179 μmol/g/10min) compared with 3) Δ-insulin in 3hPEX+PES group above that of 3hREST group (0.362 μmol/g/10min). Doubling the amount of in vivo exercise (1 h vs. 2 h) or in vitro contraction (5 tetani vs. 10 tetani) did not result in higher values for the subsequent insulin-stimulated glucose transport. The apparent additivity of the effects on insulin-stimulated glucose transport suggests that distinct mechanisms may be responsible for the enhanced insulin sensitivity after in vivo exercise or in vitro electrical stimulation.

We attempted to identify the mechanism whereby insulin-stimulated glucose transport in PEX+PES group is enhanced above that of PEX or PES group. We found that levels of 1) AS160 Thr^{642} phosphorylation, TBC1D1 PAS-phosphorylation and GLUT4 abundance found in 3hPEX+PES group, and 2) AS160 Thr^{642} phosphorylation, AMPK phosphorylation and glycogen depletion found in 0hPEX+PES group were not greater than the levels found in PEX group. Therefore, the further enhancement in

insulin sensitivity in the PEX+PES group cannot be attributed to the differences in these factors.

One possible explanation for the reversed AS160 phosphorylation in the 3hPES group (not different from the level of 3hREST group) is that a prior electrical stimulation activates the dephosphorylation of AS160 (e.g., via enhanced activation of phosphatases). Therefore, it was possible that muscles from rats that have undergone in vivo exercise prior to in vitro electrical stimulation (PEX+PES) would exhibit reversed AS160 Thr^{642} phosphorylation 3 h after contraction (i.e., in vitro contraction may interfere with the effect of in vivo exercise to persistently elevate AS160 Thr^{642} phosphorylation). Had the values for AS160 Thr^{642} phosphorylation in 3hPEX+PES group been reversed to the levels of 3hREST group, it would have provided evidence against the role of enhanced AS160 Thr^{642} phosphorylation in increased insulin-stimulated glucose transport post-exercise. However, AS160 Thr^{642} phosphorylation in the 3hPEX+PES group remained elevated above the 3hREST group, similar to the level of the 3hPEX group, consistent with the idea that AS160 Thr^{642} phosphorylation may play a role in enhanced insulin-stimulated glucose transport 3 h after exercise.

In conclusion, this study provides novel insights into the mechanisms for enhanced insulin-stimulated glucose transport with in vivo exercise or in vitro electrical stimulation. Prolonged increase in AS160 phosphorylation has been repeatedly observed in rats and humans (1, 12, 26, 28), concomitant with enhanced insulin-stimulated glucose transport in rats (1, 12) and improved insulin sensitivity in humans (28). However, the current study clearly demonstrated that the increased insulin sensitivity after in vitro electrical stimulation in serum is not because of a prolonged increase in AS160

phosphorylation (PAS or Thr^{642}). At first glance, this may be interpreted to suggest that a prolonged increase in AS160 phosphorylation is only incidental to the enhanced insulin-stimulated glucose transport post-exercise. However, the effects of in vivo exercise and in vitro electrical stimulation on a subsequent increase in insulin-stimulated glucose transport were additive, suggesting that distinct mechanisms may be responsible for increased insulin sensitivity after in vivo exercise or in vitro electrical stimulation. Therefore, enhanced AS160 phosphorylation remains a viable candidate for playing an essential role in increased insulin-stimulated glucose transport after in vivo exercise. The apparently distinct mechanisms for in vitro electrical stimulation to enhance insulin sensitivity can also be explored as a potential alternative pathway to therapeutically enhance insulin-stimulated glucose transport in skeletal muscle.

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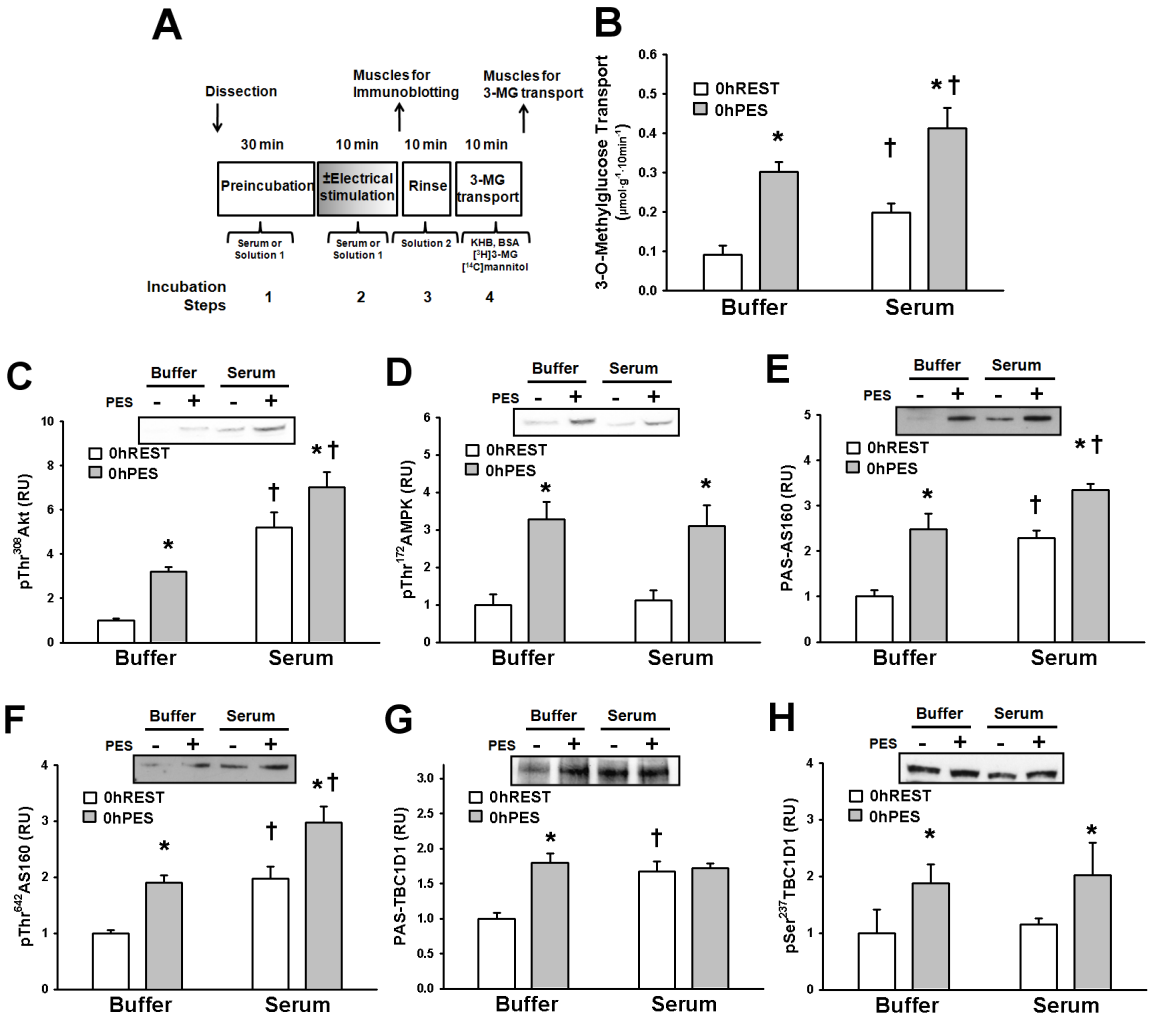


Figure 6.1

0hPES vs. 0hREST. Rat epitrochlearis muscles were incubated in either buffer or serum prior to and during in vitro electrical stimulation or resting control. A) Experimental design, B) Rate of 3-O-methylglucose (3-MG) transport, C) pThr^{308}Akt, D) pThr^{172}AMPK, E) PAS-AS160, F) pThr^{642}AS160, G) PAS-TBC1D1 and H) pSer^{237}TBC1D1. Data were analyzed with two-way ANOVA and the Student-Newman-Keuls post-hoc test. *P < 0.05 (electrical stimulation effect; post-hoc test); †P < 0.05 (serum effect; post-hoc test). Data are means ± SE, n = 4-6 per group. Open bars = resting (0hREST); grey bars = immediately after electrical stimulation (0hPES). RU = relative units.

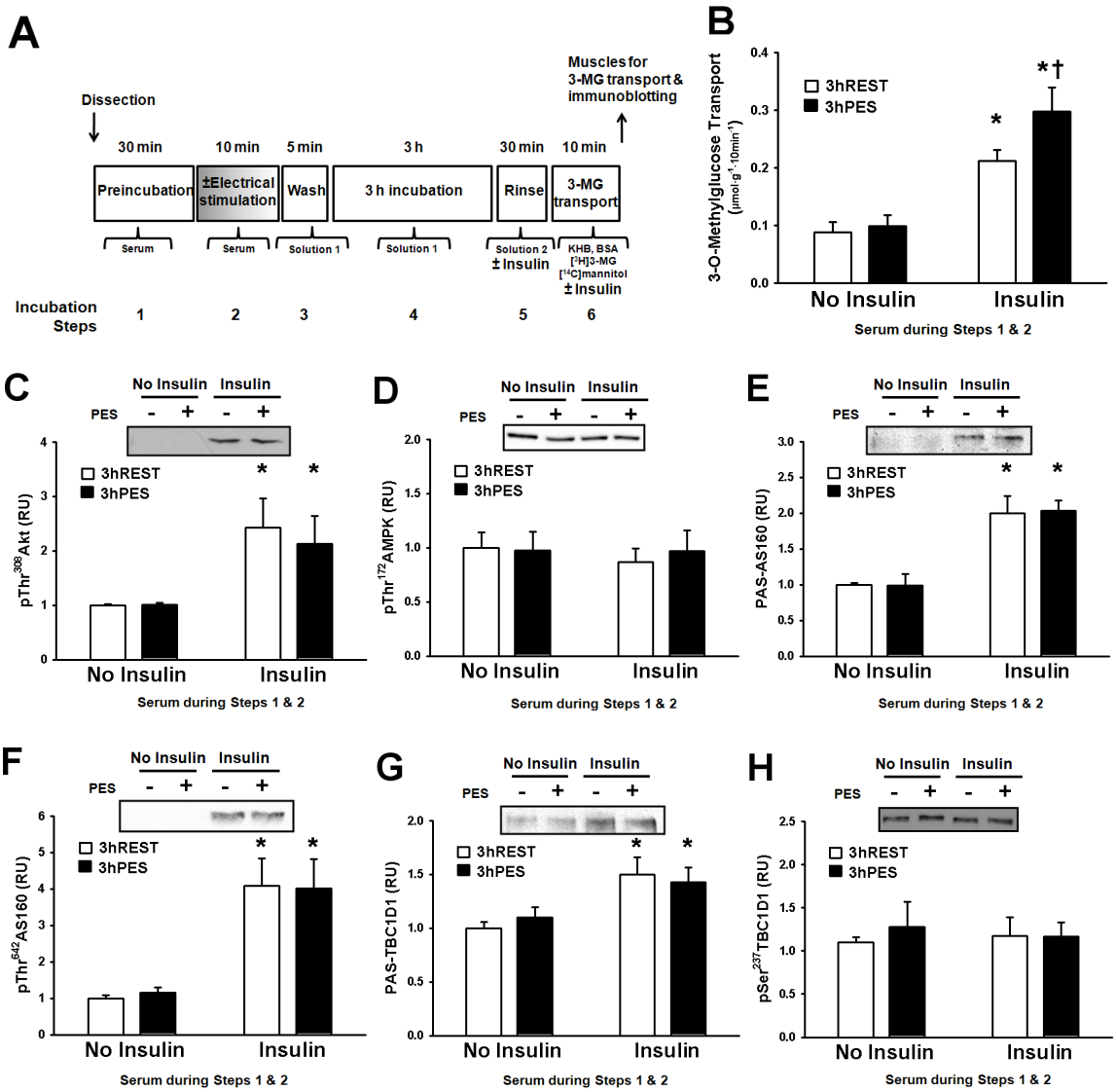


Figure 6.2

3hPES vs. 3hREST. Rat epitrochlearis muscles were incubated in serum prior to and during in vitro electrical stimulation or resting control and were subsequently incubated in buffer solution for 3 h. A) Experimental design, B) Rate of 3-O-methylglucose (3-MG) transport, C) pThr^{308}Akt, D) pThr^{172}AMPK, E) PAS-AS160, F) pThr^{642}AS160, G) PAS-TBC1D1 and H) pSer^{237}TBC1D1. Data were analyzed with two-way ANOVA and the Student-Newman-Keuls post-hoc test. *$P < 0.05$ (insulin effect; post-hoc test); †$P < 0.05$ (post-electrical stimulation effect; post-hoc test). Data are means ± SE, n = 6-12 per group. Open bars = resting in serum followed by 3 h incubation in buffer (3hREST); filled bars = in vitro electrical stimulation in serum followed by 3 h incubation in buffer (3hPES). RU = relative units.

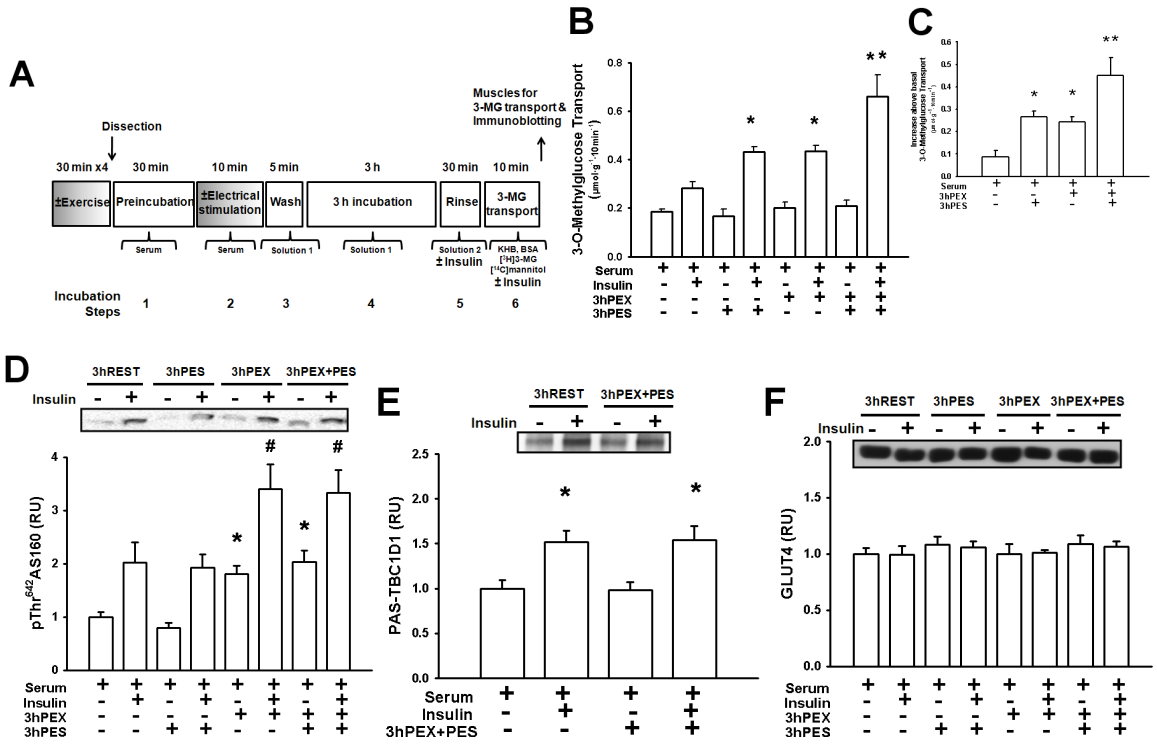


Figure 6.3

3h post-exercise+electrical stimulation (3hPEX+PES). Following 2 h of exercise bout (4 x 30 min) or sedentary, isolated rat epitrochlearis muscles were incubated in serum prior to and during in vitro electrical stimulation or resting control and were subsequently incubated in buffer solution for 3 h. A) Experimental design, B) Rate of 3-O-methylglucose (3-MG) transport; data for muscles incubated without insulin and those incubated with insulin were each analyzed with two-way ANOVA and the Student-Newman-Keuls post-hoc test. *P < 0.05 (significantly different from 3hREST); **P < 0.05 (significantly different from 3hREST, 3hPEX and 3hPES). C) Δ-insulin (increase above basal, calculated by subtracting the values for muscles incubated without insulin from the respective values of paired muscles incubated with insulin) for the rate of 3-O-methylglucose (3-MG) transport; data were analyzed with two-way ANOVA and the Student-Newman-Keuls post-hoc test. *P < 0.05 (significantly different from 3hREST); **P < 0.05 (significantly different from 3hREST, 3hPEX and 3hPES). D) pThr^{642}AS160; data for muscles incubated without insulin and those incubated with insulin were each analyzed with two-way ANOVA and the Student-Newman-Keuls post-hoc test. *P < 0.05 (significantly different from 3hREST, comparison within muscles that were incubated without insulin); $^{\#}$P < 0.05 (significantly different from 3hREST, comparison among muscles that were incubated with insulin). E) PAS-TBC1D1; data were analyzed with two-way ANOVA and the Student-Newman-Keuls post-hoc test. *P < 0.05 (significantly different from 3hREST). F) GLUT4 abundance. Data are means ± SE, n = 8-14 per group.

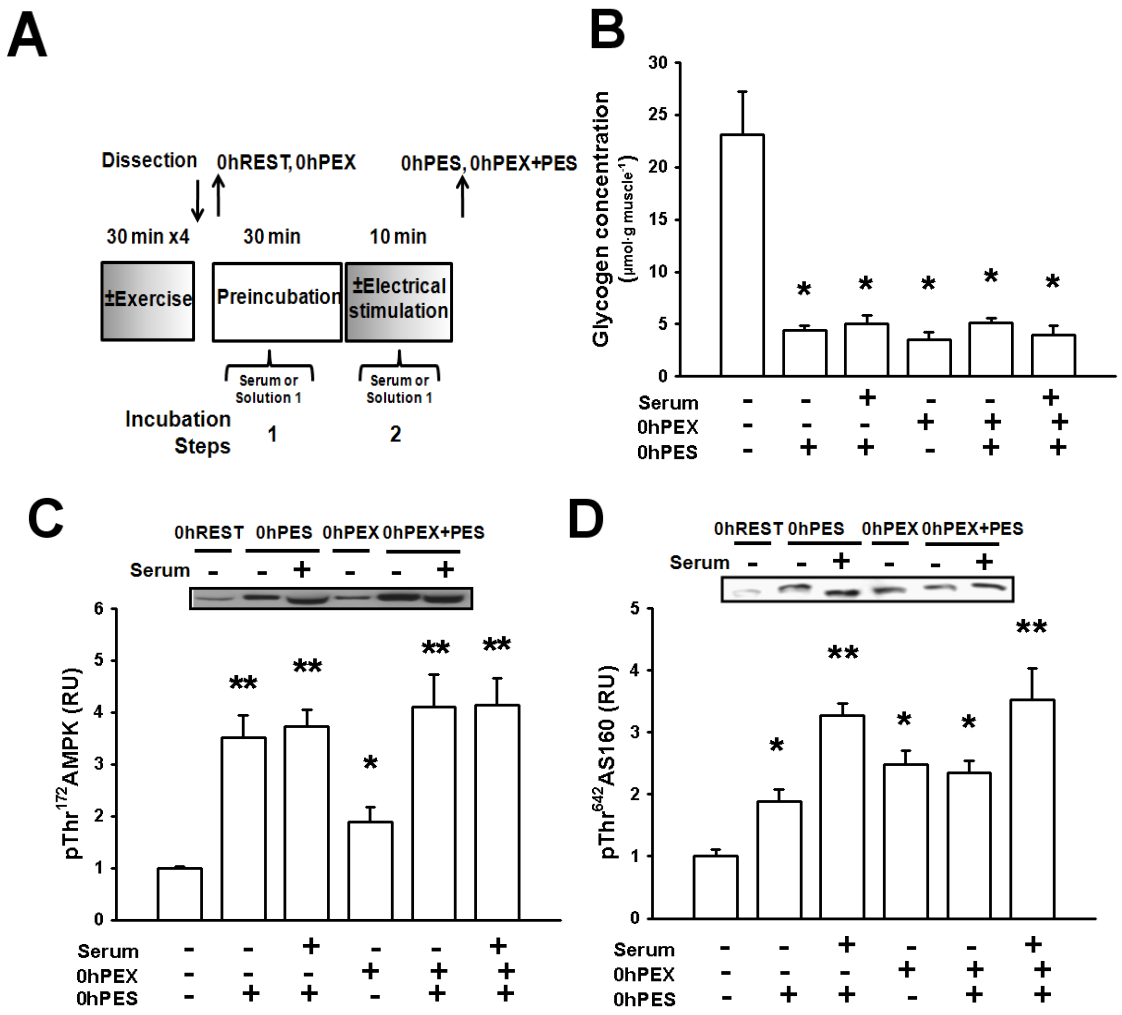


Figure 6.4

Immediately after exercise+electrical stimulation (0hPEX+PES). Following 2 h of exercise bout (4 x 30 min) or sedentary, isolated rat epitrochlearis muscles were frozen immediately or were incubated in serum or serum-free buffer prior to and during in vitro electrical stimulation or resting control and were frozen immediately after. A) Experimental design, B) Muscle glycogen concentration: data were analyzed with Kruskal-Wallis nonparametric ANOVA on ranks and the Dunn's post-hoc test. *P < 0.05 (significantly different from 0hREST). C) pThr^{172}AMPK; data were analyzed with one-way ANOVA and the Student-Newman-Keuls post-hoc test. *P < 0.05 (significantly different from 0hREST); **P < 0.05 (significantly different from 0hREST and 0hPEX). D) pThr^{642}AS160; data were analyzed with one-way ANOVA and the Student-Newman-Keuls post-hoc test. *P < 0.05 (significantly different from 0hREST); **P < 0.05 (significantly different from 0hREST, 0hPES-buffer, 0hPEX and 0hPEX/PES-buffer). Data are means ± SE, n = 6-8 per group.

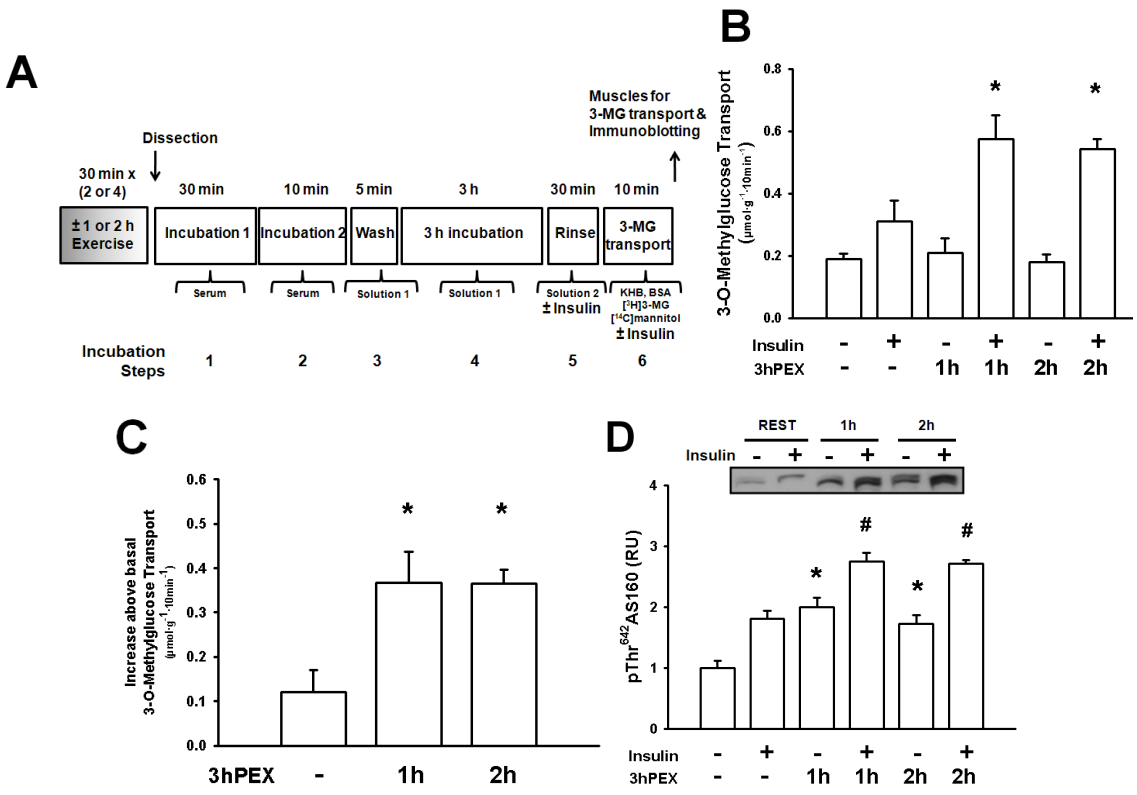


Figure 6.5

Comparison of 1 h or 2 h bout of in vivo exercise. Following 1 h of exercise bout (2 x 30 min), 2 h of exercise bout (4 x 30 min), or sedentary, rat epitrochlearis muscles were incubated in serum and were subsequently incubated in buffer solution for 3 h. A) Experimental design, B) Rate of 3-O-methylglucose (3-MG) transport; data for muscles incubated without insulin and those incubated with insulin were each analyzed with one-way ANOVA and the Student-Newman-Keuls post-hoc test. *$P < 0.05$ (significantly different from 3hREST). C) Δ-insulin (increase above basal, calculated by subtracting the values for muscles incubated without insulin from the respective values of paired muscles incubated with insulin) for the rate of 3-O-methylglucose (3-MG) transport; data were analyzed with one-way ANOVA and the Student-Newman-Keuls post-hoc test. *$P < 0.05$ (significantly different from 3hREST). D) $pThr^{642}AS160$; data for muscles incubated without insulin and those incubated with insulin were each analyzed with one-way ANOVA and the Student-Newman-Keuls post-hoc test. *$P < 0.05$ (significantly different from 3hREST, comparison within muscles that were incubated without insulin); $^{\#}P < 0.05$ (significantly different from 3hREST, comparison among muscles that were incubated with insulin). Data are means ± SE, n = 6 per group.

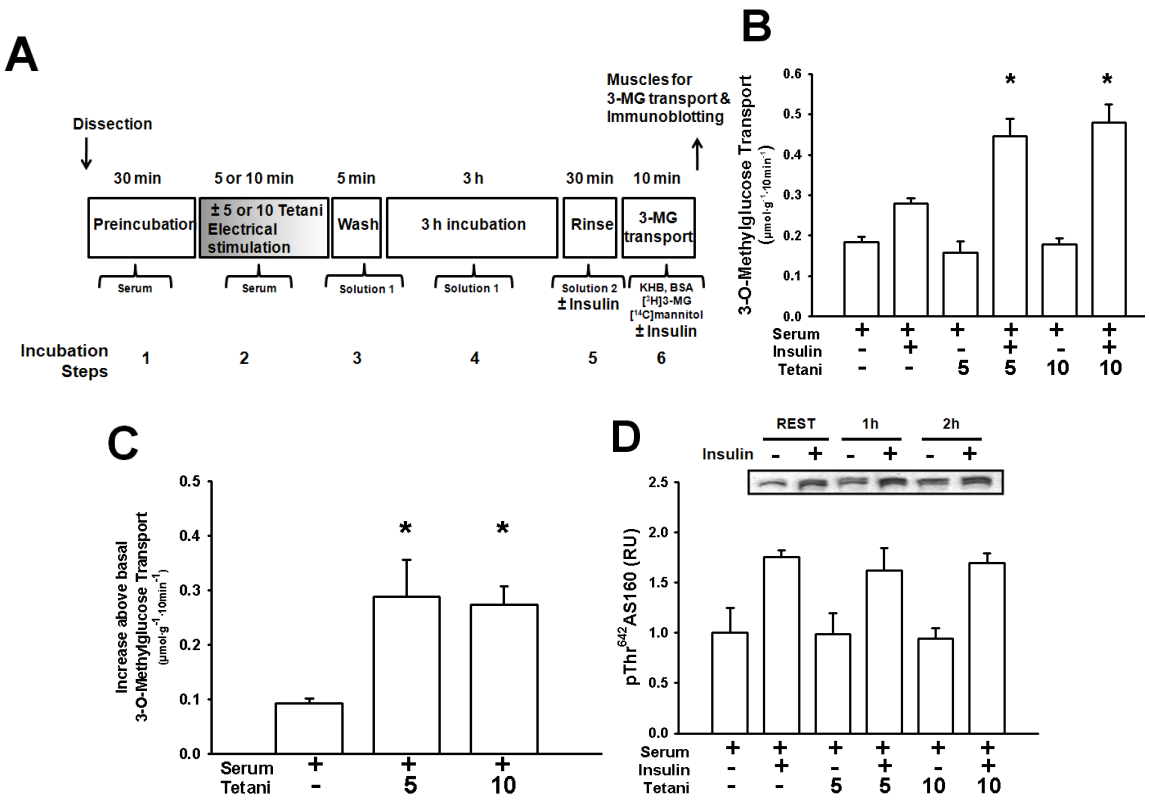
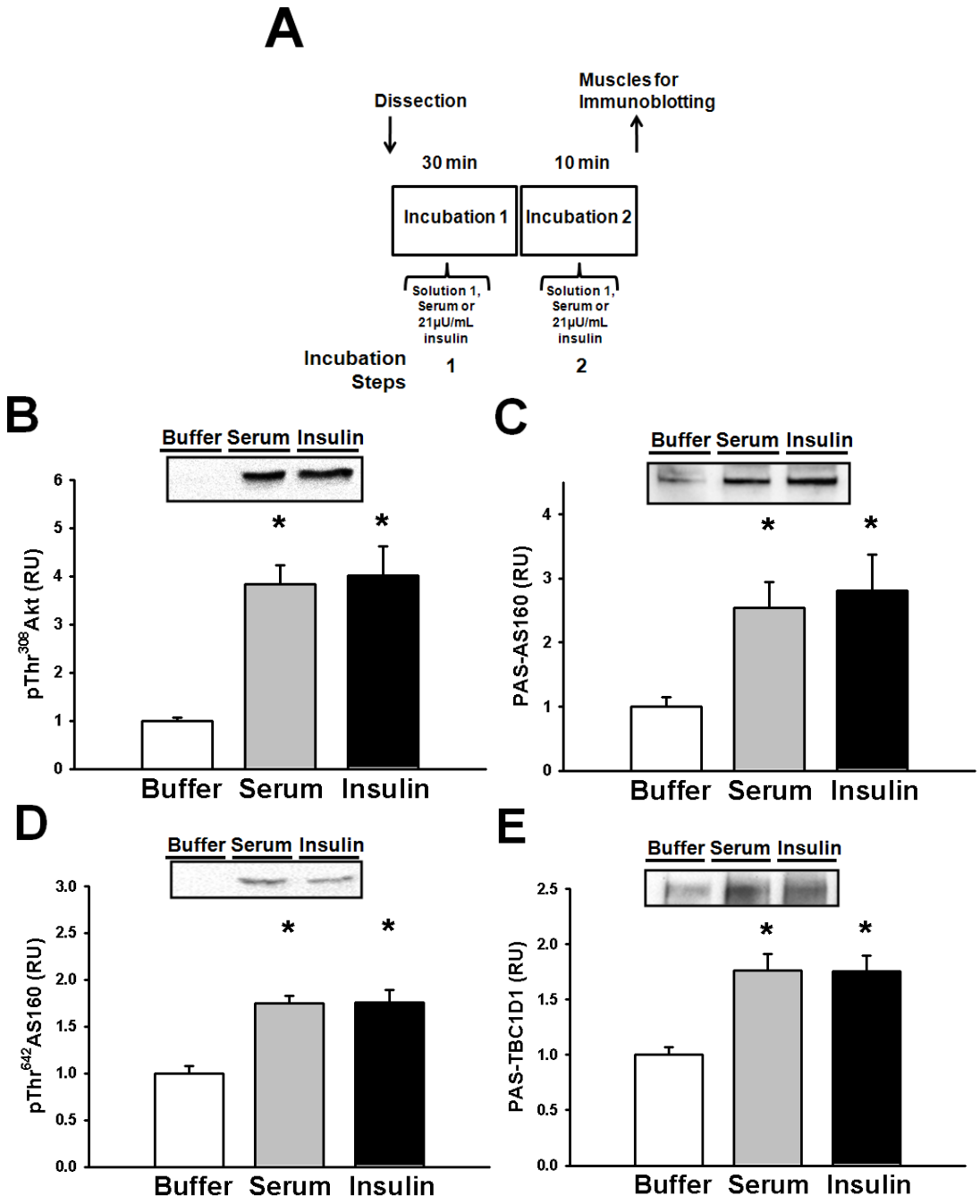


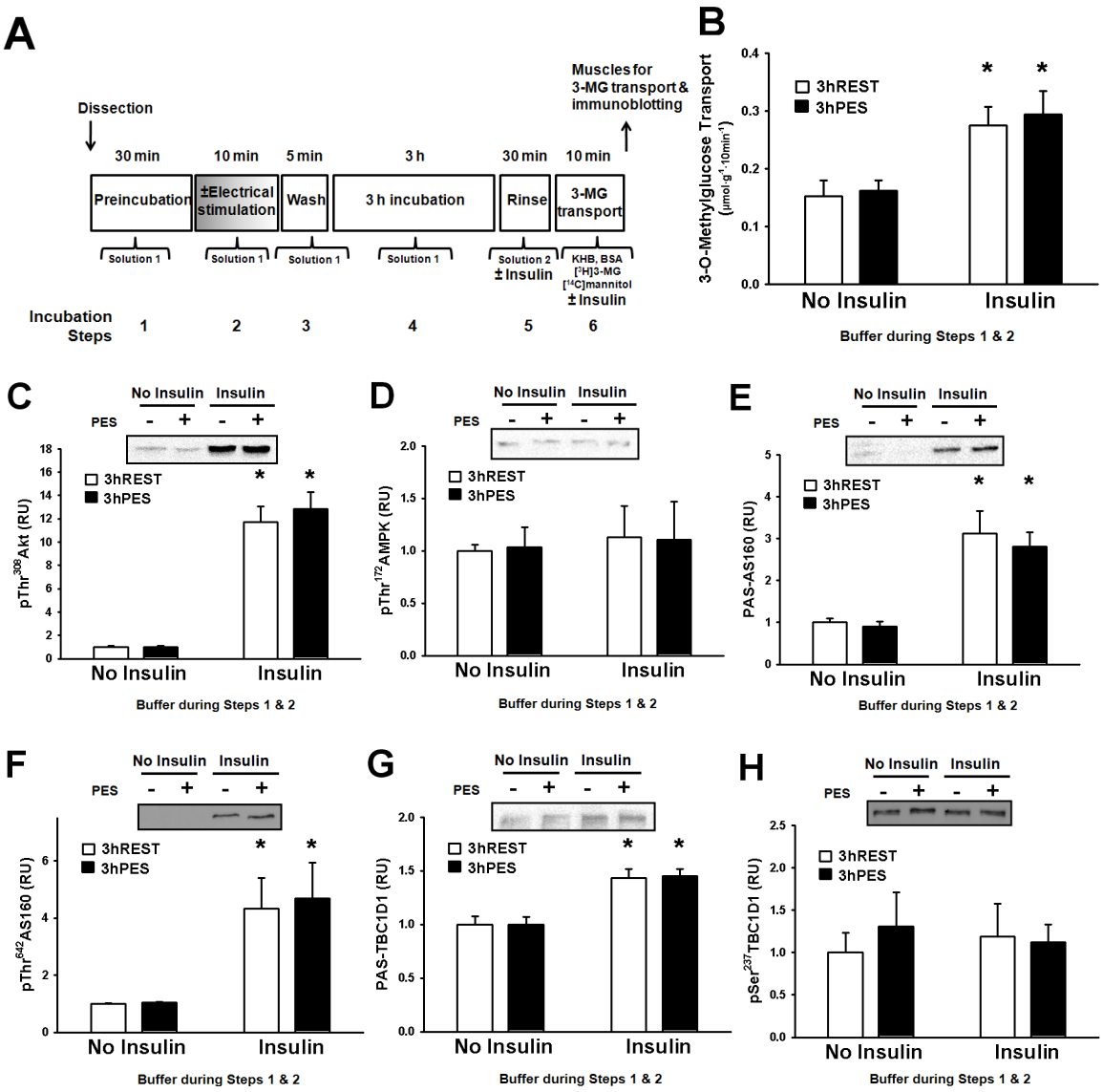
Figure 6.6

Comparison of 5 or 10 tetani in vitro electrical stimulation. Isolated rat epitrochlearis muscles were incubated in serum prior to and during in vitro electrical stimulation (5 vs. 10 tetani) or resting control and were subsequently incubated in buffer solution for 3 h. A) Experimental design, B) Rate of 3-O-methylglucose (3-MG) transport; data for muscles incubated without insulin and those incubated with insulin were each analyzed with one-way ANOVA and the Student-Newman-Keuls post-hoc test. *$P < 0.05$ (significantly different from 3hREST). C) Δ-insulin (increase above basal, calculated by subtracting the values for muscles incubated without insulin from the respective values of paired muscles incubated with insulin) for the rate of 3-O-methylglucose (3-MG) transport; data were analyzed with one-way ANOVA and the Student-Newman-Keuls post-hoc test. *$P < 0.05$ (significantly different from 3hREST). D) $pThr^{642}AS160$; data for muscles incubated without insulin and those incubated with insulin were each analyzed with one-way ANOVA and the Student-Newman-Keuls post-hoc test. Data are means ± SE, n = 5-6 per group.



Supplemental Figure 6.S1

Serum or 21 μU/mL insulin incubation. Rat epitrochlearis muscles were incubated in either serum-free buffer, serum or 21 μU/mL of insulin (equal to serum insulin concentration). A) Experimental deisgn, B) $pThr^{308}Akt$, C) PAS-AS160, D) $pThr^{642}AS160$ and E) PAS-TBC1D1. Data were analyzed using one-way ANOVA and the Student-Newman-Keuls post-hoc test. *$P < 0.05$ (significantly different from buffer group). Data are means ± SE, n = 4-6 per group. Open bars = incubated in serum-free buffer; grey bars = incubated in serum; filled bars = incubated in 21 μU/mL of insulin. RU = relative units.



Supplemental Figure 6.S2

3hPES vs. 3hREST (in serum-free buffer). Rat epitrochlearis muscles were incubated in serum-free buffer prior to and during in vitro electrical stimulation and were subsequently incubated in another buffer solution for 3 h. A) Experimental design, B) Rate of 3-O-methylglucose (3-MG) transport, C) pThr^{308}Akt, D) pThr^{172}AMPK, E) PAS-AS160, F) pThr^{642}AS160, G) PAS-TBC1D1 and H) pSer^{237}TBC1D1. Data were analyzed with two-way ANOVA and the Student-Newman-Keuls post-hoc test. *P < 0.05 (insulin effect; post-hoc test). Data are means ± SE, n = 6-12 per group. Open bars = resting in serum-free buffer followed by 3 h incubation in buffer (3hREST); filled bars = in vitro electrical stimulation in serum-free buffer followed by 3 h incubation in buffer (3hPES). RU = relative units.

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CHAPTER VII

DISCUSSION

Focus of this Discussion

This discussion will 1) summarize the key findings of each study, 2) review relevant studies that were published during and after completion of the studies included in the dissertation, 3) describe how the findings in this dissertation advance the current understanding of the role that AS160 and TBC1D1 phosphorylation play on the regulation of skeletal muscle glucose transport after muscle contractile activity, 4) identify new questions raised by the results in the dissertation, 5) propose a brief research plan for a future experiment, and 6) provide overall conclusions.

Summary of Key Findings

Results from this dissertation provide a number of novel insights into the cellular mechanisms involved in the regulation of insulin-independent and insulin-dependent glucose transport after a single bout of exercise. Previous research established a need to investigate the role that phosphorylation of AS160 and TBC1D1 may play on the regulation of glucose transport during and after exercise. Accordingly, the studies in this dissertation focused on exploring the possibility that AS160 or TBC1D1 phosphorylation may be involved in increased insulin-independent and insulin-dependent glucose

transport after a single bout of in vivo exercise or in vitro contractile activity. A summary of the key findings is provided below.

Study 1: *Contraction-stimulated Glucose Transport in Rat Skeletal Muscle is Sustained despite Reversal of Increased PAS-phosphorylation of AS160 and TBC1D1*

Isolated rat epitrochlearis muscles were stimulated to contract (2 ms twitch, 2 Hz) for various lengths of time (5, 10, 20, 40 or 60 min) or were used for resting control. One of the paired muscles was used for the measurement of 3-*O*-methylglucose transport whereas the contralateral muscle was frozen immediately and used for immunoblotting.

- Consistent with previous results, pAkt (Ser^{473} and Thr^{308}), pGSK3 (an Akt substrate), pCaMKII and pSRF (a CaMKII substrate) responded to contraction transiently, whereas pAMPK and pACC (an AMPK substrate) were increased throughout the duration of contraction.
- Using immunoblotting, a contraction-responsive phosphorylated protein band, PAS-150, was identified and found to correspond with PAS-TBC1D1, a second contraction-responsive phosphorylated protein band, PAS-160, was found to correspond with PAS-AS160.
- PAS-150 (TBC1D1) and PAS-160 (AS160) responded to contraction transiently (peaked at 20 min), despite sustained AMPK activation for 60 min.
- Glucose transport was increased throughout the duration of contraction, despite reversal of the increases in PAS-150 (TBC1D1) and PAS-160 (AS160).
- Glucose transport significantly correlated with pAMPK, but not with pGSK3, pCaMKII, PAS-150 or PAS-160.

Study 2: *Inhibition of contraction-stimulated AMPK Inhibits Contraction-stimulated Increases in PAS-TBC1D1 and Glucose Transport without Altering PAS-AS160 in Rat Skeletal Muscle*

Isolated rat epitrochlearis muscles were incubated in either wortmannin or Compound C prior to and during in vitro electrical stimulation (2 ms twitch, 2Hz, 20 min), insulin-stimulation (2 mU/mL, 20 min) or AICAR stimulation (2 mM, 40 min). One of the paired muscles was used for the measurement of 3-*O*-methylglucose transport whereas the contralateral muscle was frozen immediately and was used for immunoprecipitation and/or immunoblotting.

- The PI3K inhibitor wortmannin eliminated the insulin-mediated increases in pThrAkt, pGSK3, PAS-160 (AS160), PAS-150 (TBC1D1) and glucose transport.
- The PI3K inhibitor wortmannin eliminated contraction-stimulated increases in pThrAkt, pGSK3 and PAS-AS160 without affecting contraction's elevation in pAMPK, pACC, PAS-TBC1D1 or glucose transport.
- The AMPK inhibitor Compound C eliminated contraction-stimulated increases in pACC and PAS-TBC1D1 and partially inhibited (62%) contraction-stimulated increase in glucose transport, without affecting pThrAkt, pGSK3 or PAS-AS160.
- Neither wortmannin nor Compound C affected tension development or pCaMKII with contraction.
- Compound C eliminated AICAR-stimulated glucose transport without affecting insulin-stimulated glucose transport.

Study 3: *Increased AS160 Phosphorylation, but not TBC1D1 Phosphorylation, with Increased Post-exercise Insulin Sensitivity in Rat Skeletal Muscle*

Male Wistar rats were randomly assigned to post-exercise (PEX) or sedentary (SED) treatment. Rats were either: 1) anesthetized immediately post-exercise without access to food after exercise (0hPEX and 0hSED); 2) anesthetized 3 h post-exercise without access to food after exercise (3hPEX and 3hSED); 3) anesthetized 3 h post-exercise with unlimited access to food after exercise (3hPEX-chow and 3hSED-chow); or 4) anesthetized 27 h post-exercise without access to food after exercise (27hPEX and 27hSED). Epitrochlearis muscles were dissected and were either frozen immediately or were incubated for the measurement of 3-*O*-methylglucose transport (without insulin for 0hPEX and 0hSED groups and with or without insulin for other groups). Frozen muscles were used for immunoprecipitation, immunoblotting or glycogen measurement.

- Post-exercise glucose transport (measured in the absence of insulin) consistently tracked with PAS-TBC1D1 (i.e., both increased at 0h, and both reversed at 3h and 27hPEX).
- The reversal of post-exercise glucose transport (measured in the absence of insulin) at 3 and 27PEX was not accompanied by reversal of PAS-AS160 or pThr^{642}AS160.
- The post-exercise increase in insulin-stimulated glucose transport consistently tracked with enhanced PAS-AS160, pThr^{642}AS160 and pThrAkt (i.e., both increased at 3hPEX and 27hPEX, and both reversed at 3hPEX-chow).
- The post-exercise increase in insulin-stimulated glucose transport was not accompanied by enhanced PAS-TBC1D1 or Akt activity.

- Glycogen depletion coincided with enhanced insulin-stimulated glucose transport at 3hPEX and 3hPEX-chow, but not at 27hPEX.

Study 4: *Additive Effects of In Vivo Exercise and In Vitro Contraction on Insulin-stimulated Glucose Transport by Rat Skeletal Muscle*

Male Wistar rats were randomly assigned to: 1) resting (REST); 2) post-electrical stimulation (in vitro electrical stimulation, ± serum, 0.1 ms twitch, 100 Hz train for 10 s, 10 trains, 10 min: PES); 3) post-exercise (in vivo exercise: PEX); or 4) post-exercise and electrical stimulation (in vivo exercise followed by in vitro electrical stimulation: PEX+PES) groups. After in vivo exercise or in resting controls, epitrochlearis muscles were dissected and were either immediately frozen (0hSED or 0hPEX) or were incubated in rat serum prior to during in vitro electrical stimulation. Subsequently muscles were either frozen immediately (0hPES or 0hPEX+PES) or were incubated in buffer solution for 3 h prior to being incubated with 3-*O*-methylglucose transport (3hREST, 3hPEX, 3hPES or 3hPEX+PES). Frozen muscles were used for immunoprecipitation, immunoblotting or glycogen measurement.

- The increase in glucose transport with electrical stimulation (without insulin) consistently tracked with increases in PAS-AS160, pThr^{642}AS160, PAS-TBC1D1, pSer^{237}TBC1D1, pAMPK and pThrAkt (all were increased at 0hPES and all were reversed at 3hPES).
- The post-contraction increase in insulin-stimulated glucose transport 3 h after electrical stimulation in serum was not accompanied by PAS-AS160, pThr^{642}AS160, PAS-TBC1D1, pSer^{237}TBC1D1, pAMPK or pThrAkt (with or

without insulin, values at 3hPES-serum was not different from values at 3hREST).

- Doubling the amount of exercise (1 h vs. 2 h) or electrical stimulation (5 tetani or 10 tetani) did not result in a higher value for the subsequent insulin-stimulated glucose transport and pThr^{642}AS160.
- The effects of exercise (PEX) and electrical stimulation (PEX) on subsequent increase in insulin-stimulated glucose transport were additive in muscles that were electrically stimulated in vitro after in vivo exercise (PEX+PES).
- The enhanced insulin-stimulated glucose transport in the PEX+PES group above that of either PEX or PES alone was not explained by differences from PEX or PES groups for: 1) pThr^{642}AS160 at 3hPEX+PES; 2) PAS-TBC1D1 at 3hPEX+PES; 3) GLUT4 abundance at 3hPEX+PES; 4) pThr^{642}AS160 at 0hPEX+PES; 5) pAMPK at 0hPEX+PES; or 6) glycogen concentration at 0hPEX+PES.

Relevant Studies Published after the Dissertation Proposal

Several relevant studies were published on the regulation of AS160 or TBC1D1 during and after completion of the experiments in this dissertation.

David James' group further investigated the role that association of AS160 to GLUT4 vesicles may play on the regulation of GLUT4 translocation in cultured cells (32). Consistent with previous results (22, 23), AS160 associated with GLUT4 vesicles in the basal condition. Interestingly, expression of a GLUT4/AS160-RabGAP (the peptide sequence of AS160's RabGAP domain fused to the C terminus of GLUT4) fusion

protein by 3T3-L1 cells prevented insulin-stimulated GLUT4 translocation, and this inhibitory effect was eliminated when cells were expressed a GLUT4/AS160-RabGAP-R/A (GLUT4 fused with constitutively inactive RabGAP of AS160) fusion protein (basal GLUT4 translocation in either of these cells was not different compared to wildtype cells). Expression of the AS160-RabGAP domain alone (without GLUT4) had no detectable effect on basal or insulin-stimulated cell-surface GLUT4. These results suggested that association of AS160 active RabGAP domain with GLUT4 vesicles is sufficient to restrain insulin-stimulated GLUT4 translocation. As reported previously (22), insulin-stimulated phosphorylation of AS160 and its dissociation from GLUT4 vesicles. Cells that expressed the GLUT4/AS160 fusion protein (GLUT4 fused with full length AS160) underwent normal insulin-stimulated GLUT4 translocation. Furthermore, the GLUT4/AS160 fusion protein in these insulin-stimulated cells was phosphorylated on the AS160 Thr^{642} site. Together, these results suggested that insulin-stimulated release of AS160 from GLUT4 vesicles is not required for GLUT4 translocation. Moreover, in L6 myotubes, many agonists that stimulate GLUT4 translocation to the cell-surface membranes (AICAR, adiponectin, berberine, IL-6 or endothelin-1) appeared to require AS160 for this action (expression of AS160-4P mutant partially GLUT4 translocation) but, unlike insulin, these agonists did not result in a detectable release of AS160 from GLUT4 vesicles into the cytosol. It remains possible that GLUT4 translocation induced with these agonists are mediated through dissociation of TBC1D1, and not AS160, from GLUT4 vesicles. It is currently unknown whether in vivo exercise results in: 1) a dissociation of AS160 from GLUT4 vesicles immediately after exercise, and 2) if a prolonged increase in AS160 phosphorylation several hours after exercise is coupled with

a sustained dissociation of AS160 to GLUT4 vesicles. An exercise induced increase in AS160 phosphorylation is unlikely to be essential for insulin-independent increase in glucose transport (1, 3, 9, 13, 15, 34, 36), but it remains possible that it is sufficient for AS160 dissociation from GLUT4 vesicles. Although in vivo exercise may be insufficient to fully translocate AS160-associated GLUT4 vesicles to the cell-surface membranes, AS160 phosphorylation (with or without dissociation from GLUT4 vesicles) may eliminate AS160's inhibitory effect and render GLUT4 vesicles more susceptible to subsequent insulin-triggered translocation. Thus, sustained phosphorylation of AS160 from GLUT4 could potentially explain the mechanism for enhanced insulin-stimulated glucose transport several hours after exercise.

Tim McGraw's group provided further insights into the mechanisms whereby insulin stimulates membrane dissociation of AS160 in 3T3-L1 adipocytes (17). To measure membrane associated AS160, they used a quantitative microscopy assay in which cells were detergent permeabilized before fixation to release soluble cytosolic contents (29). Consistent with previous reports (22, 32), the amount of AS160 associated with plasma membrane was reduced by insulin stimulation. Importantly, the effect of insulin on AS160 plasma membrane association was blunted by RNAi knockdown of Akt2, but not Akt1. Furthermore, consistent with the idea that Akt2, but not Akt1, is responsible for AS160 phosphorylation with insulin stimulation, knockdown of Akt2, but not Akt1, blunted insulin-stimulated phosphorylation of AS160. Therefore, Akt2 specifically regulates the phosphorylation and plasma membrane release of AS160 in response to insulin, providing insights into the molecular explanation for Akt isoform-specific signaling to GLUT4. In contrast, results from Laurie Goodyear's laboratory (21)

showed that: 1) increased AS160 PAS phosphorylation with in vitro contraction was significantly reduced in EDL muscles that were incubated in PI3K inhibitor wortmannin (that resulted in complete inhibition of contraction-stimulated phosphorylation of pan-Akt, presumably including both Akt1 and Akt2), whereas 2) increased AS160 PAS or Thr^{642} phosphorylation with in situ contraction was not reduced in EDL muscles from Akt2 knockout mice. Therefore, by deduction, phosphorylation of AS160 during contraction may occur in Akt1-dependent, but not Akt2-dependent, manner. This difference may suggest that insulin and contraction stimulate phosphorylation of different pools of AS160. Consistent with this idea, the effects of insulin and contraction on AS160 PAS and Thr^{642} phosphorylation were additive (21). In turn, in conjunction with results (32) interpreted in the previous paragraph, it seems conceivable that Akt2-mediated phosphorylation of AS160 with insulin results in GLUT4 translocation, whereas Akt1-mediated phosphorylation of AS160 with contraction results in sequestering GLUT4 vesicles to a location more susceptible to insulin-triggered translocation. A caveat to this idea is that mechanism for AS160 phosphorylation has not been investigated with in vivo exercise (only with in vitro or in situ contraction), and it remains possible that other kinases such as AMPK may be involved in exercise-induced phosphorylation of AS160.

Evidence suggests that there may be multiple AS160 binding proteins that are involved in the regulation of insulin-stimulated GLUT4 translocation. In addition to 14-3-3 that has been previously described (10, 27), Rip11 (37), RUVBL2 (40) and ClipR-59 (8) have all been implicated to be important for insulin-stimulated GLUT4 translocation through its interaction with AS160. Each of these proteins associates with AS160, and

knockdown of each of these proteins resulted in the inhibition of insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (8, 37, 40). Details for the mechanisms that explain how these proteins regulate GLUT4 translocation are not yet fully elucidated. It would also be important to explore the possibility that some of these proteins might also be involved in exercise stimulated increase in insulin-independent and insulin-dependent increase in GLUT4 translocation.

Sharma et al. (30) investigated the effect of a transient insulin incubation on AS160 phosphorylation in rat epitrochlearis muscles. Incubation of epitrochlearis muscle in 300 μU/mL of insulin for 30 min followed by a subsequent incubation of the muscle in the absence of insulin resulted in a rapid reversal of AS160 phosphorylation (75% reduction after 10 min, and complete reversal to no insulin level after 20 min). Although the rapid loss of AS160 phosphorylation after insulin removal might seem to be predictable, this expectation was less certain in the context of earlier observation that, after a bout of exercise, AS160 phosphorylation can remain increased (1, 11, 14, 15, 31). It is unclear if the differing results after insulin and exercise are because of differences inherent to the two stimuli, or other differences in the experimental designs of the study compared to these earlier studies. For example, the insulin stimulation was performed in vitro whereas in vivo exercise was used in the earlier studies. In the context of a subsequent increase in insulin-stimulated glucose transport, the lack of prolonged increase in AS160 phosphorylation after a transient insulin incubation concomitant with the known lack of subsequent increase in insulin sensitivity is consistent with the idea that sustained increase in AS160 phosphorylation may contribute to increased insulin-stimulated glucose transport (also supported by data from Study 1, 3 and 4) (12, 14, 15).

Geiger et al. reported that incubation of isolated skeletal muscle in serum and supraphysiologic dose of insulin resulted in a subsequent increase in insulin-stimulated glucose transport (16). Therefore, it seems plausible that the reversal of insulin-stimulated AS160 phosphorylation would be delayed if serum was added during the initial, transient insulin incubation. However, the results from Study 4 of this dissertation (14) suggest that the effect that in vitro contraction in serum has on a subsequent increase in insulin sensitivity may be different from the effect of in vivo exercise. Not only was the AS160 phosphorylation not elevated in the muscles that had undergone prior contraction in serum (in contrast to the sustained increase in AS160 phosphorylation post-exercise), the effect of prior contraction and exercise on insulin-stimulated glucose transport was additive, supporting the idea that there are distinct mechanisms that stimulate increased insulin sensitivity under in vitro contraction in serum or in vivo exercise. The role that AS160 phosphorylation may play on increased insulin-stimulated glucose transport after the incubation with insulin and serum is unresolved.

Gus Lienhard's group followed up on their previous reports (4, 28) on the role that TBC1D1 phosphorylation plays in insulin-stimulated GLUT4 translocation in 3T3-L1 adipocytes (25). In their previous studies (4, 28), insulin was unable to stimulate GLUT4 translocation in cells that overexpressed wildtype TBC1D1. In their more recent study, by reducing the level of ectopic TBC1D1 expression (WT), insulin significantly increased cell-surface GLUT4 content (25). Furthermore, cells that overexpressed TBC1D1 with Ser/Thr to Ala mutation on three phosphorylation sites (3P; Thr^{590}, Thr^{499} and Ser^{489} that in WT becomes phosphorylated in response to insulin) had lower insulin-stimulated cell-surface GLUT4 when compared to WT cells, consistent with the idea that

insulin-stimulated phosphorylation of TBC1D1 can increase GLUT4 translocation. Nonetheless, because overexpression of wildtype AS160 had no influence on insulin-stimulated GLUT4 translocation (whereas low level of TBC1D1 overexpression still lowered cell-surface GLUT4 by ~60% in insulin-stimulated cells compared to cells with empty vector expression), at least in 3T3-L1 adipocytes, insulin appears to be more effective in regulating increased GLUT4 translocation by increasing AS160 phosphorylation than by increasing TBC1D1 phosphorylation.

In addition to the evidence provided in Study 2 of this dissertation (13), recent reports support the idea that contraction stimulates TBC1D1 in AMPK-dependent manner. In C2C12 myotubes that generate highly developed contractile activity (due to manipulation of intracellular calcium transients with electric pulse stimulation), excitation induced contractile activity resulted in an activation of AMPK concomitant with TBC1D1 phosphorylation on the Ser^{237} site (24).

Consistent with the results from rat epitrochlearis muscles in Study 2, wortmannin had no effect on contraction-stimulated increase in PAS-TBC1D1, $pThr^{596}$TBC1D1 or $pSer^{237}$TBC1D1 in isolated EDL muscles from wildtype mice (26). In addition, isolated EDL muscles from AMPK kinase dead mice lacked a contraction-stimulated increase in PAS-TBC1D1, $pThr^{596}$TBC1D1, $pSer^{237}$TBC1D1 or 14-3-3 binding to TBC1D1 (26), consistent with the idea that AMPK phosphorylates TBC1D1 on these sites during contraction. However, insulin-stimulated PAS-TBC1D1 or $pThr^{596}$TBC1D1 was also significantly lower in muscles from AMPK kinase dead mice. Phosphorylation of TBC1D1 on Ser^{237} was not significantly elevated in response to insulin. The implication of these results is unclear, but the authors interpreted that basal phosphorylation of

TBC1D1 (on Ser^{237}, Thr^{596} or other residues that are yet unidentified) by an AMPK-dependent mechanism is required for insulin to exert its effect on PAS-TBC1D1 or $pThr^{596}$TBC1D1. The effects of insulin and contraction (incubated in insulin prior to contraction) were not additive on PAS-TBC1D1 (consistent from the results of Study 4 of this dissertation (14)) and $pThr^{596}$TBC1D1. The lack of additive effects on PAS-TBC1D1 and $pThr^{596}$TBC1D1 is in contrast with results that the effects of insulin and contraction on AS160 PAS or Thr^{642} phosphorylation were additive (21). The simplest interpretation for the apparent differences is that insulin and contraction stimulates phosphorylation of: 1) AS160 from distinct pools, and 2) TBC1D1 from a shared pool. This idea is supported by the evidence that: 1) the effects of insulin and contraction on AS160 PAS or Thr^{642} phosphorylation are additive (21), and 2) Akt1 may phosphorylate AS160 during contraction (21) whereas Akt2 phosphorylates AS160 with insulin (17). Under a number of circumstances, binding of 14-3-3 to TBC1D1 closely matched that of $pSer^{237}$TBC1D1 in mouse EDL muscle (26). Binding of 14-3-3 has been suggested to be important for the regulation of its RabGAP activity (5, 22), supporting the idea that phosphorylation of TBC1D1 on $pSer^{237}$ site may a play role in the regulation of GLUT4 translocation.

Isolated rat epitrochlearis muscles that were stimulated to contract in the presence of a myosin II ATPase inhibitor, N-benzyl-p-toluenesulfonamide (BTS), had significantly lower pAMPK, pACC and PAS-TBC1D1, without significantly affecting PAS-AS160 compared to the muscles that were stimulated to contract in the vehicle (2). In addition, contraction-stimulated glucose transport was significantly lower in the muscles with BTS (2), providing additional evidence that contraction may stimulate glucose transport via

the AMPK-TBC1D1 axis. Together, these data provide evidence that contraction stimulates phosphorylation of TBC1D1 in AMPK-dependent manner.

In addition to reports that a single bout of endurance exercise results in a prolonged increase in AS160 phosphorylation (1, 11, 14, 15, 31), a single bout of resistance exercise in humans also resulted in a subsequent increase in AS160 phosphorylation (9). In contrast, AS160 phosphorylation (determined using PAS antibody) was not increased immediately after the resistance exercise, despite increased in vivo leg glucose uptake. These results are consistent with the notion that increased AS160 phosphorylation on PAS sites is not essential for insulin-independent increase in glucose transport during exercise.

Treebak et al. reported site-specific phosphorylation of AS160 determined 4 h post-exercise (60 min of dynamic one-legged knee extensor exercise at ~80-100% of peak workload, contralateral leg was used for the resting control) in human vastus lateralis biopsies (35). In contrast to findings in rat epitrochlearis muscles (1, 14, 15) and previous studies in humans (11, 31), PAS-AS160 was not different between muscles that have undergone prior exercise and the sedentary controls (35). Phosphorylation at Thr^{642} and Ser^{666} sites of AS160 were also not different between exercised muscles and the sedentary controls. However, phosphorylation at Ser^{318}, Ser^{341} and Ser^{751} (borderline significance at Ser^{588}, $P = 0.09$) sites of AS160 were enhanced in exercised muscles compared to the sedentary controls. Although the explanation for the differences in the results from two studies for PAS-AS160 and $pThr^{642}$AS160 after exercise is uncertain, there is a great deal of evidence for a persistent increase in AS160 in skeletal muscle after acute exercise.

How the Studies in this Dissertation Fill Gaps in Current Understanding

The studies in this dissertation were designed to provide insights into the roles that AS160 or TBC1D1 play in the regulation of skeletal muscle glucose transport after exercise and electrically stimulated contraction. Between the identification of AS160 (in 2002) and TBC1D1 (in 2007) and the proposal of this dissertation (September 24, 2008), there were only 17 publications that included original experimental data on AS160 and/or TBC1D1 with contraction or exercise. In the subsequent year, this number increased by ~50% (to 26 publications, as of November 23, 2009). It is apparent that there is a growing interest in the role that AS160 or TBC1D1 play in the regulation of skeletal muscle glucose transport after exercise.

The major goal of Study 1 was to characterize the time course of AS160 and TBC1D1 phosphorylation during contraction of isolated skeletal muscle. It was hypothesized that, consistent with the idea that Akt phosphorylates AS160 and AMPK phosphorylates TBC1D1 during contraction, AS160 PAS-phosphorylation would increase transiently (concomitant with transient activation of Akt) and TBC1D1 PAS-phosphorylation would have a sustained increase (concomitant with sustained increases in AMPK activity and glucose transport). AS160 PAS-phosphorylation responded transiently, consistent with the idea that: 1) Akt phosphorylates AS160 on PAS-detectable sites during contraction, and 2) AS160 PAS-phosphorylation is not required for a sustained increase in contraction-stimulated glucose transport. In contrast to the hypothesis, TBC1D1 PAS-phosphorylation also responded transiently, providing evidence for a disconnect between TBC1D1 PAS-phosphorylation with: 1) TBC1D1's

upstream kinase AMPK, or 2) TBC1D1's downstream target glucose transport. It remains possible that TBC1D1 PAS-phosphorylation results in sequestering of TBC1D1 to a subcellular compartment inaccessible by AMPK. Recently, studies in HEK-293 cells suggested that PAS antibody mainly recognizes phosphorylation on Thr^{596}, but not Ser^{237}, on TBC1D1 (5). Furthermore, phosphorylation on Ser^{237}, but not PAS or Thr^{596} phosphorylation, appeared to better coincide with increase in insulin-independent glucose transport with contraction (14, 26). Therefore, it is possible that phosphorylation of TBC1D1 on Ser^{237} remained elevated throughout 60 min of contraction. It also remains possible that the initial increase in TBC1D1 PAS-phosphorylation is sufficient for a sustained increase in glucose transport.

The major goal of Study 2 was to use inhibitors of key signaling proteins to investigate the mechanisms whereby contraction stimulates phosphorylation of AS160 or TBC1D1. It was hypothesized that: 1) the PI3K inhibitor wortmannin would inhibit AS160 PAS-phosphorylation without affecting TBC1D1 PAS-phosphorylation or glucose transport, and 2) the AMPK inhibitor Compound C would inhibit TBC1D1 PAS-phosphorylation and glucose transport, but not AS160 PAS-phosphorylation. In support of the hypotheses, AS160 PAS-phosphorylation was eliminated using wortmannin without affecting TBC1D1 PAS-phosphorylation or glucose transport, consistent with the idea that Akt-dependent phosphorylation of AS160 on PAS-sites are not essential for contraction-stimulated glucose transport. Also in support of the hypotheses, TBC1D1 PAS-phosphorylation was eliminated using Compound C without affecting AS160 PAS-phosphorylation, consistent with the idea that AMPK phosphorylates TBC1D1 on PAS-sites during contraction. Concomitantly, Compound C partially (62%) inhibited glucose

transport, consistent with the idea that AMPK contributes to increasing glucose transport through TBC1D1 PAS-phosphorylation. Subsequent research has shown that among multiple phosphorylation sites on TBC1D1, increase in phosphorylation on Ser^{237} site coincided most with 14-3-3 binding which has been implicated for TBC1D1's function on regulating glucose transport (5, 26). Furthermore, contraction-stimulated increase in TBC1D1 phosphorylation (PAS, Thr^{596} or Ser^{237}) or 14-3-3 binding capacity was completely inhibited in mouse EDL from AMPKα2 knockout mice (26), consistent with the idea that AMPK-dependent processes regulate TBC1D1 function with in vitro contraction. At the time when Study 2 was being conducted, a phospho-specific antibody for $pSer^{237}$TBC1D1 was not available. We have since obtained this antibody and confirmed that phosphorylation on this site is: 1) increased immediately after in vitro contraction when insulin-independent glucose transport is increased, and 2) reversed to the resting level 3 h after in vitro contraction when insulin-independent glucose transport is also reversed (Study 4) (14). It would be useful to confirm the results in this study by examining whether Compound C completely inhibits contraction-stimulated $pSer^{237}$TBC1D1.

Together, Study 1 and Study 2 provide evidence that TBC1D1 PAS-phosphorylation, but not AS160 PAS-phosphorylation, may be important for insulin-independent increase in glucose transport during and immediately after in vitro contraction. It is still possible that: 1) phosphorylation sites not recognized by PAS antibody, 2) binding of calmodulin, 14-3-3 or other proteins (Rip11, RUVBL2, ClipR-59, etc.) are important for the contraction effect on insulin-independent glucose transport, or 3) inhibitors used in the studies may have had non-specific effects towards other proteins

involved in the regulation of glucose transport. Genetic or pharmacological manipulation directly on TBC1D1 would be useful to further identify the role that TBC1D1 phosphorylation may play on contraction- or exercise-stimulated glucose transport. Although results from Study 1 showed that the timecourse for AS160 and TBC1D1 phosphorylation coincided with each other, Study 2 clearly showed that, by using two different inhibitors, phosphorylation of these two proteins can be teased apart, suggesting that there are distinct mechanisms that phosphorylate AS160 and TBC1D1 during in vitro contraction.

The major goal of Study 3 was to probe the functional importance of AS160 or TBC1D1 phosphorylation under four experimental conditions that were known to vary in their influence on insulin-independent glucose transport (or exercise-stimulated glucose transport) and insulin-dependent glucose transport after exercise (post-exercise increase in insulin sensitivity). It was hypothesized that: 1) increased TBC1D1 phosphorylation would be found in protocols with increased insulin-independent glucose transport but not in protocols with enhanced insulin-dependent glucose transport; 2) the protocols with enhanced insulin-dependent glucose transport after exercise would be accompanied by increased AS160 phosphorylation; and 3) that protocols without enhanced insulin-dependent glucose transport after exercise would not be characterized by elevated AS160 phosphorylation. Consistent with the hypotheses and with the idea that TBC1D1 phosphorylation is important for exercise-stimulated glucose transport, TBC1D1 PAS-phosphorylation was increased immediately after exercise when insulin-independent glucose transport was elevated, but was reversed at 3 h and 27 h post-exercise when the insulin-independent increase in glucose transport had reversed. Also in support with the

hypotheses, TBC1D1 PAS-phosphorylation was not enhanced at 3 h or 27 h post-exercise conditions, consistent with the idea that TBC1D1 phosphorylation does not contribute to post-exercise increase in insulin-stimulated glucose transport. In contrast to the results with TBC1D1, but also in support of the hypotheses, increased AS160 phosphorylation (PAS-AS160 and pThr^{642}AS160) consistently tracked with increased insulin-stimulated glucose transport in post-exercise conditions. In contrast, glycogen depletion did not always coincide with increased insulin-stimulated glucose transport. Together, these findings provide evidence that AS160 PAS or Thr^{642} phosphorylation, but not TBC1D1 PAS-phosphorylation may be important for the post-exercise increase in insulin-stimulated glucose transport.

The major goal of Study 4 was to determine if the increased insulin-stimulated glucose transport after in vitro electrical stimulation of isolated skeletal muscle in the presence of serum was accompanied by enhanced AS160 phosphorylation, similar to results after in vivo exercise (Study 3). It was hypothesized that: 1) in isolated skeletal muscles that were stimulated to contract in serum-free buffer (which is known to not result in a subsequent increase insulin-stimulated glucose transport), would not induce a persistent increase in AS160 phosphorylation at 3 h post-electrical stimulation, and 2) in isolated skeletal muscles that were stimulated to contract in serum (which is known to result in a subsequent increase in insulin-stimulated glucose transport) would induce both enhanced AS160 phosphorylation and insulin-stimulated glucose transport 3 h after the electrical stimulation. Contrary to the hypotheses, regardless of the presence of serum, skeletal muscles that were stimulated to contract in vitro did not have a persistently enhanced AS160 phosphorylation (measured with PAS-AS160 and pThr^{642}AS160) at 3 h

post-electrical stimulation, despite elevated insulin-stimulated glucose transport after contraction in serum. TBC1D1 phosphorylation (measured with PAS-TBC1D1 and pSer^{237}TBC1D1) was also unaltered 3 h after electrical stimulation, regardless of the presence of serum during contraction. Importantly, insulin-stimulated glucose transport in muscles from rats that had undergone in vivo exercise prior to in vitro electrical stimulation was greater than that of muscles after exercise or electrical stimulation alone. The enhanced insulin-stimulated glucose transport in exercise+electrical stimulation group above that in exercise or electrical stimulation groups was not attributable to greater phosphorylation of AS160 or TBC1D1 or by greater glycogen depletion in muscles that underwent both in vivo exercise and in vitro contraction. Although not conclusive, the additivity of prior electrical stimulation and prior exercise on the subsequent increase in insulin-stimulated glucose transport (using exercise and electrical stimulation protocols that appeared to be maximally effective) suggests that mechanisms whereby prior electrical stimulation (in serum) increases insulin sensitivity are distinct from the mechanisms whereby prior in vivo exercise increases insulin sensitivity. Therefore, it remains possible that the persistently enhanced AS160 phosphorylation plays a role in the post-exercise increase insulin-stimulated glucose transport. It is also important to note that prolonged increase in pThr^{642}AS160 (and concomitant increase in insulin-stimulated glucose transport) was found in muscles that were dissected immediately after exercise and were incubated in the buffer solution for 3 h (whereas muscles were dissected 3 h after exercise in Study 3). Therefore, the mechanisms for a sustained increase in AS160 phosphorylation and insulin-stimulated glucose transport

appears to be intrinsic to changes occur in muscles during exercise, independent of external factors in vivo after exercise.

Together, Study 3 and Study 4 provide evidence that AS160 phosphorylation (PAS or Thr^{642}), but not TBC1D1 phosphorylation (PAS or Ser^{237}), may play a role in insulin-dependent increase in glucose transport in rat skeletal muscles at 3 and 27 h after exercise. Furthermore, in multiple conditions, the level of glycogen depletion did not match with enhanced insulin-stimulated glucose transport, suggesting that glycogen depletion alone does not explain the post-exercise increase in insulin-stimulated glucose transport. They also provide data consistent with the idea (from Study 2) that TBC1D1 Ser^{237} phosphorylation may be important for the insulin-independent increase in glucose transport.

In addition, results from Study 4 suggest that in vitro electrical stimulation and in vivo exercise may stimulate a subsequent increase in insulin-stimulated glucose transport through distinct mechanisms. In vivo exercise and in vitro contraction differ many ways, including presence of blood flow and pressure changes, presence of extracellular hormones and metabolites (which may change during exercise), neuromuscular stimulation vs. field electrical stimulation, stimulation pattern (twitch length, twitch frequency, tetanus frequency and duration) and isometric contraction vs. physiologic (shortening and lengthening) contraction. In vivo exercise and in vitro contraction results in the activation of many similar signaling events. However, studies in this dissertation also indicate that there are some noteworthy differences in several signaling events after in vivo exercise and in vitro contraction (Table 7.1). The distinct mechanisms for in vivo exercise and in vitro contraction to enhance insulin-stimulated glucose transport may be

attributable to (but not limited to) these differences. It is also plausible that exercise or electrically stimulated contraction may induce insulin-independent glucose transport through distinct mechanisms. Hence, one must be cautious when interpreting data from skeletal muscle in vitro electrical stimulation (including Study 1 and Study 2) as a model for in vivo exercise. Genetic or direct pharmacological manipulation of AS160 or TBC1D1 would be useful to further identify the role that these RabGAP proteins may play in the exercise stimulated increase in insulin-independent and insulin-dependent glucose transport.

In summary, studies in this dissertation and those that have been published by others suggest the following for the roles that AS160 or TBC1D1 phosphorylation play on insulin-independent and insulin-dependent increase in glucose transport after in vivo exercise and in vitro contraction:

1. AS160 phosphorylation (on Thr^{642} or PAS sites) in skeletal muscle was elevated immediately after in vivo exercise (1, 7, 11, 15, 19, 20, 31, 34). However, in multiple exercise conditions, elevated AS160 Thr^{642} or PAS phosphorylation could be uncoupled from increase in insulin-independent glucose transport (1, 9, 15, 34). Results from studies using in vitro contraction provide further evidence that contraction-stimulated PAS-phosphorylation of AS160 was not essential for contraction-stimulated glucose transport (2, 12, 13, 21, 36). Taken together, there is strong evidence that suggests that AS160 phosphorylation, at least on PAS sites, are not essential for insulin-independent increase in glucose transport in skeletal muscle after in vivo exercise.

2. TBC1D1 phosphorylation (on PAS sites) in skeletal muscle was elevated immediately after in vivo exercise (15). Unlike AS160 phosphorylation that remained

elevated for several hours post-exercise, TBC1D1 PAS-phosphorylation reversed to resting level 3 h after exercise, concomitant with the reversal of insulin-independent increase in glucose transport (15). Immediately after in vitro contraction, TBC1D1 phosphorylation became elevated on PAS, $Ser^{237}$and Thr^{596} sites (2, 12-14, 26, 33). Phosphorylation of Ser^{237} TBC1D1 was reversed to the resting level 3 h after contraction when contraction-stimulated glucose transport was reversed (14). Results from studies using pharmacological inhibitors with in vitro contraction provided evidence that, in multiple conditions, contraction-stimulated PAS-phosphorylation of TBC1D1 consistently tracked with contraction-stimulated glucose transport (2, 13). In skeletal muscles that were deficient in AMPKα2, which has been implicated for increasing insulin-independent glucose transport, TBC1D1 phosphorylation on PAS, $Ser^{237}$and Thr^{596} sites were completely inhibited. In mouse EDL and cultured cells, TBC1D1's 14-3-3 binding capacity (which has been suggested to play a role in the regulation of GLUT4 translocation) closely tracked with phosphorylation of TBC1D1 on Ser^{237} site (5, 26). Taken together, it seems reasonable to suggest that phosphorylation of TBC1D1 on Ser^{237} may play a role in insulin-independent increase in glucose transport in skeletal muscle immediately after in vivo exercise.

3. Unlike other insulin-signaling steps (IR tyrosine kinase activity, IRS tyrosine phosphorylation, IRS associated PI3K activity, Akt Ser^{473} phosphorylation, TBC1D1 PAS-phosphorylation, etc.) (1, 6, 15, 38, 39), that were not enhanced in skeletal muscles several hours after exercise, AS160 PAS or Thr^{642} phosphorylation in skeletal muscles remained elevated up to 27 h after exercise (1, 15, 31, 35). AS160 PAS or Thr^{642} phosphorylation was elevated at 3 and 27 h after exercise (fasted after exercise) when

insulin-stimulated glucose transport also remained elevated, whereas AS160 PAS or Thr^{642} phosphorylation and insulin-stimulated glucose transport both reversed to the resting level when rats were fed with carbohydrate-rich chow for 3 h after exercise (15). It would be important to further pursue the role that AS160 phosphorylation (especially on Thr^{642} site) in post-exercise increase in insulin-stimulated glucose transport. Enhanced insulin-stimulated glucose transport observed in muscles that were stimulated to contract in serum occured independent of AS160 phosphorylation (at least on PAS or Thr^{642} site) and through mechanisms that may be distinct from that of in vivo exercise.

Future Directions

The studies in this dissertation have revealed novel aspects of the mechanisms involved in insulin-independent or insulin-dependent increase in glucose transport with in vitro contraction and in vivo exercise. Results from experiments using inhibitors with in vitro electrical stimulation (Study 2) suggest that AMPK-dependent phosphorylation of TBC1D1 (on Ser^{237}), but not Akt-dependent phosphorylation of AS160 (on Thr^{642}), is important for insulin-independent increase in glucose transport. However, evidence from Study 4 suggests that results from in vitro electrical stimulation may not always coincide with results in vivo exercise, thus, it would be important to study the mechanism for insulin-independent glucose transport with in vivo exercise. In contrast, results from Study 3 and Study 4 suggest that enhanced AS160 phosphorylation may be important for the post-exercise increase in insulin-dependent glucose transport. However, interpretation of these results are largely dependent on associations between protein phosphorylation and glucose transport and do not provide direct evidence for the role of

AS160 on the post-exercise increase in insulin-dependent glucose transport. The results of these studies raised the following questions:

1) Are/is AS160 and/or TBC1D1 phosphorylation essential for the normal increase in insulin-independent glucose transport after in vivo exercise?

2) Are/is AS160 and/or TBC1D1 phosphorylation essential for the post-exercise increase in insulin-dependent glucose transport?

No studies have yet identified the effect of AS160 or TBC1D1 genetic manipulation on the insulin-independent and insulin-dependent glucose transport in skeletal muscle after in vivo exercise. The following section will outline a brief proposal of experiments aimed at answering these questions using mice that are genetically deficient in AS160 or TBC1D1.

Proposal for Future Research

Specific Aims

Studies with pharmacological inhibitors suggest that, in isolated skeletal muscles that are stimulated to contract in vitro, TBC1D1 phosphorylation (but not AS160 phosphorylation) may play a role in the insulin-independent increase in glucose transport. In contrast, mechanisms for the insulin-independent glucose transport with in vivo exercise are uncertain. On the other hand, AS160 phosphorylation (but not TBC1D1 phosphorylation) appears to be important for the post-exercise increase in insulin-dependent glucose transport. However, no studies have directly assessed the role of AS160 or TBC1D1 on the post-exercise increase in insulin-dependent glucose transport.

Therefore, the proposed research will investigate the effects of AS160 or TBC1D1 deletion on insulin-independent and insulin-dependent glucose transport after exercise. To minimize the possibility of compensatory mechanisms as a result of whole body knockout, mice with a muscle-specific tamoxifen-inducible AS160 or TBC1D1 deletion will be created.

Specific Aim 1: *Determine if insulin-stimulated glucose uptake is affected in mouse skeletal muscles with inducible deletion of gene AS160 or TBC1D1*

Hypothesis 1-A: AS160 deletion will result in a partial reduction in insulin-stimulated glucose uptake in skeletal muscle.

Hypothesis 1-B: TBC1D1 deletion will have no effect on insulin-stimulated glucose uptake in skeletal muscle.

Specific Aim 2: *Determine if the exercise-stimulated glucose uptake is affected in mouse skeletal muscles with inducible deletion of the gene AS160 or TBC1D1.*

Hypothesis 2-A: AS160 deletion will have no effect on exercise-stimulated glucose uptake in skeletal muscle.

Hypothesis 2-B: TBC1D1 deletion will result in a partial reduction in exercise-stimulated glucose uptake in skeletal muscle.

Specific Aim 3: *Determine if the post-exercise increase in insulin-stimulated glucose uptake is affected in mouse skeletal muscles with inducible deletion of the gene AS160 or TBC1D1.*

Hypothesis 3-A: AS160 deletion will result in an elimination of post-exercise increase in insulin-stimulated glucose uptake in skeletal muscle.

Hypothesis 3-B: TBC1D1 deletion will have no effect on post-exercise increase in insulin-stimulated glucose uptake in skeletal muscle.

Research Design and Methods

Mice with flanked loxP sites on AS160 or TBC1D1 gene will be crossed with mice with muscle creatine kinase (MCK) specific tomoxifen-inducible Cre-ER. Subsequently, the offspring containing the desired genotype will be backcrossed to C57/B6 mice for 10 generations. At age 4 wks, mice will be either injected with tamoxifen or vehicle for two weeks (4 intraperitoneal injections over the course of a week). Muscle-specific inducible AS160 knockout mice ($MCK^{Cre\text{-}ER}$ $AS160^{loxP}$ tamoxifen injected: $mAS160^{-/-}$) or TBC1D1 knockout mice ($MCK^{Cre\text{-}ER}$ $TBC1D1^{loxP}$ tamoxifen injected: $mTBC1D1^{-/-}$) and their respective controls not injected with tamoxifen ($mAS160^{+/+}$ or $TBC1D1^{+/+}$) will be used for this study.

Mice (male, 6 wks) will be randomly assigned to treadmill running exercise (15-25 m/min for 1 h (18), PEX) or sedentary control (SED). Mice will be either anesthetized immediately (0hPEX or 0hSED) or 3 h after exercise (3hPEX or 3hSED). Epitrochlearis (EPI) and soleus (SOL) muscles will be dissected and are either frozen immediately or will be incubated for the measurement of 2-deoxyglucose uptake (without insulin immediately after exercise, and with or without 50 μU/mL insulin 3 h after exercise). Frozen muscles will be used to determine glycogen concentration or for

imunoprecipitation/immunoblotting (protein abundance and phosphorylation for AS160 on Thr^{642} and TBC1D1 on Ser^{237}).

Anticipated Results

A) Inducing a muscle-specific AS160 knockout will result in (compared to tomaxifen uninjected controls): 1) a slight increase in basal glucose uptake; 2) partial reduction in insulin-stimulated glucose uptake; 3) no effect on insulin-independent glucose uptake immediately after exercise; and 4) a lack of post-exercise increase in insulin-stimulated glucose uptake 3 h after exercise.

B) Inducing a muscle-specific TBC1D1 knockout will result in (compared to tomaxifen uninjected controls): 1) a slight increase in basal glucose uptake; 2) no effect on insulin-stimulated glucose uptake; 3) a partial reduction in insulin-independent glucose uptake immediately after exercise; and 4) no effect on post-exercise increase in insulin-stimulated glucose uptake 3 h after exercise.

Interpretation

The hypothesis that AS160, but not TBC1D1, is important for a portion of insulin-stimulated increase in glucose uptake would be supported if insulin-stimulated glucose uptake is partially suppressed in $mAS160^{-/-}$ mice but not in $mTBC1D1^{-/-}$ mice. In contrast, the hypothesis that TBC1D1, but not AS160, is important for insulin-independent glucose transport after exercise would be supported if insulin-independent glucose uptake is suppressed in $mTBC1D1^{-/-}$ mice but not in $mAS160^{-/-}$ mice. Furthermore, the hypothesis that AS160, but not TBC1D1, is important for post-exercise

increase in insulin-dependent glucose transport would be supported if insulin-dependent glucose uptake is suppressed in mAS160$^{-/-}$ mice but not in mTBC1D1$^{-/-}$ mice.

It is conceivable that deletion of AS160 will result in a complete reduction of insulin-stimulated glucose uptake. In this case, the interpretation would be that AS160 is essential for any insulin-stimulated increase in glucose uptake, and that insulin-stimulated AS160-independent mechanisms, without AS160, are insufficient to increase glucose uptake. In addition, if AS160 deletion results in a lack of insulin-stimulated increase in glucose uptake, the lack of post-exercise increase in insulin-stimulated glucose transport in mAS160$^{-/-}$ would not be a definitive evidence for a specific role of AS160 in post-exercise increase in insulin sensitivity.

Potential Pitfalls and Alternative Experiment

Although the possibility of inducing compensatory mechanisms is minimized by inducing deletion in a tissue specific manner rather than on whole body level, and also by inducing deletion after the neonatal and early development rather than inducing in embryo, it remains possible that knocking out AS160 or TBC1D1 may produce unanticipated changes secondary to the effect of deletion. Deletion of these proteins may result in increased expression of other key signaling proteins in the regulation of glucose transport. In this context, it would be important to test whether AS160 deletion results in increased expression of TBC1D1, and TBC1D1 deletion results in increased expression of AS160. Other proteins such as GLUT4, GLUT1, insulin receptor, Akt and AMPK abunadance will also be measured. It would also be important to examine whether: 1) body weight, 2) food intake, 3) voluntary activity, 4) exercise capacity, 5) resting and

exercise VO_2 and RER and 6) glucose and insulin tolerance, 7) muscle mass, 8) glycogen and intramuscular triglycerides content and 9) mitochondrial enzyme are altered in these mice. For example, if exercise capacity of mTBC1D1-/- mice was found to be compromised, it would complicate the interpretation of results after in vivo exercise. In this case, mice can be compared after running on treadmill at the same relative intensity (%VO_2max) in addition to comparing them after the exercise of same absolute intensity (Watts).

Alternatively, instead of knocking out AS160 or TBC1D1 from all skeletal muscles in the body, deletion can be isolated in a single skeletal muscle by using in vivo electroporation. Accordingly, one EDL muscle per mouse will be subjected to shRNA-mediated knockdown of AS160 or TBC1D1 using an in vivo electroporation (contralateral muscle will be electroporated with scrambled shRNA). The effect of AS160 or TBC1D1 deletion on insulin-stimulated glucose transport, exercise-stimulated glucose transport, and post-exercise increase in insulin sensitivity could be assessed in these muscles.

Overall Conclusions

In conclusion, this dissertation has provided novel insights (Fig. 7.1) into the mechanisms for: 1) insulin-independent increase in skeletal muscle glucose transport during and immediately after in vitro contraction, and 2) insulin-dependent increase in skeletal muscle glucose transport 3 and 27 h after exercise. The results in the studies suggest that: 1) TBC1D1 phosphorylation (on PAS sites and/or Ser^{237} site), but not AS160 phosphorylation (on PAS sites including Thr^{642}), may be important for insulin-

independent increase in glucose transport (after in vivo exercise or in vitro contraction) in rat skeletal muscle; and 2) AS160 phosphorylation (on PAS sites including $pThr^{642}$), but not TBC1D1 phosphorylation (on PAS sites and/or Ser^{237} site), may be important for insulin-dependent increase in glucose transport (after in vivo exercise but not in vitro contraction) in rat skeletal muscle. It also provided evidence that distinct mechanisms may regulate insulin-stimulated glucose transport after in vivo exercise or in vitro electrical stimulation. AS160 and TBC1D1, two RabGAP proteins that have been implicated in the regulation of GLUT4 translocation, each becomes phosphorylated in response to in vitro contraction or in vivo exercise. Nonetheless, the mechanisms whereby in vitro contraction or in vivo exercise stimulates AS160 or TBC1D1 phosphorylation and the role that they play on the regulation of GLUT4 translocation appear to be distinct. Further clarification of the influence of exercise on AS160 and TBC1D1 function depends on further studying their regulation, including identifying each protein's site-specific phosphorylation, interactions with other proteins (e.g., 14-3-3) and/or subcellular localization.

	Electrical Stimulation vs. Rest	Exercise vs. Rest
Immediately PES or PEX (no insulin)		
Glucose Transport	↑ 10 x 10 s tetanus ↑ 10, 20, 40, 60 min twitch	↑ 120 min swim
pThr^{172}AMPK	↑ 10 x 10 s tetanus ↑ 10, 20, 40, 60 min twitch	↑ 120 min swim
pThr^{308}Akt	↑ 10 x 10 s tetanus ↔ 10, 20, 40, 60 min twitch	↔ 120 min swim
PAS-AS160^{A}, PAS-160^{B} or pThr^{642}AS160^{C}	↑ 10 x 10 s tetanus^{AC} ↑ 10 min twitch^{B} ↑ 20 min twitch^{AB} ↔ 40, 60 min twitch^{B}	↑ 120 min swim^{AC}
PAS-TBC1D1^{D}, PAS-150^{E} or pSer^{237}TBC1D1^{F}	↑ 10 x 10 s tetanus^{DF} ↑ 10 min twitch^{E} ↑ 20 min twitch^{DE} ↔ 40, 60 min twitch^{E}	↑ 120 min swim^{D}
Glycogen	↓ 10 x 10 s tetanus	↓ 120 min swim
3 hours PES or PEX		
Δ-insulin glucose transport	↑ 10 x 10 s tetanus (serum) ↔ 10 x 10 s tetanus (buffer)	↑ 60, 120 min swim
pThr^{172}AMPK	↔ 10 x 10 s tetanus	↔ 120 min swim
pThr^{308}Akt	↔ 10 x 10 s tetanus	↑ 120 min swim (+ insulin only)
PAS-AS160^{A} or pThr^{642}AS160^{C}	↔ 10 x 10 s tetanus^{AC}	↑ 60 min swim^{C} ↑ 120 min swim^{AC} (± insulin)
PAS-TBC1D1^{D} or pSer^{237}TBC1D1^{F}	↔ 10 x 10 s tetanus^{DF}	↔ 120 min swim^{D}
GLUT4 abundance	↔ 10 x 10 s tetanus	↔ 120 min swim

Table 7.1
Comparisons of signaling events and glucose transport after in vitro electrical stimulation and in vivo exercise. The table summarizes results from the 4 studies in this dissertation. PES: post-electrical stimulation (in serum or buffer unless specified), PEX: post-exercise, REST: resting control. ↑: increased compared to resting control, ↔: unchanged compared to resting control, ↓: decreased compared to resting control. A: PAS-AS160, B: PAS-160, C: pThr^{642}AS160, D: PAS-TBC1D1, E: PAS-150, F: pSer^{237}TBC1D1.

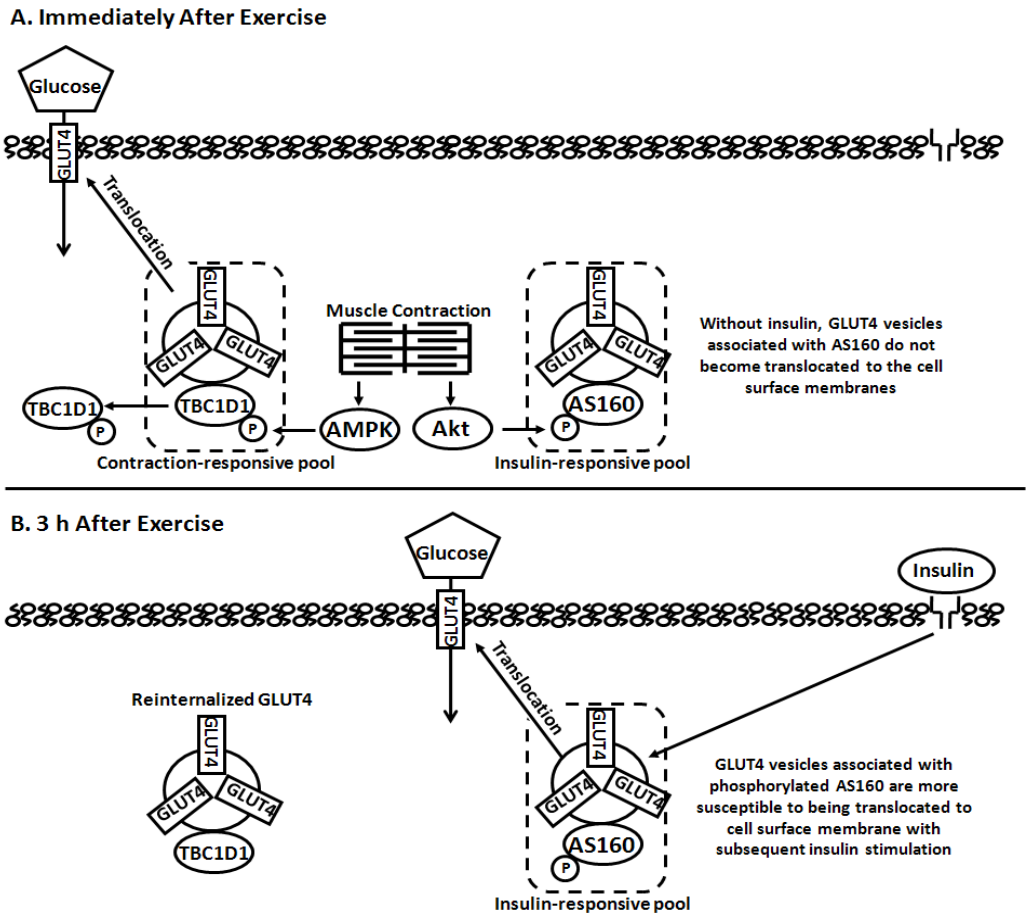

Figure 7.1
Working Hypothesis: Roles of TBC1D1 Phosphorylation on Contraction-stimulated Glucose Transport and AS160 Phosphorylation on Post-exercise Increase in Insulin-stimulated Glucose Transport. For clarity, the figure does not depict mechanisms other than AS160 and TBC1D1 phosphorylation that may influence glucose transport. A) With in vitro contraction, AMPK phosphorylates TBC1D1 on Ser^{237} site, whereas Akt phosphorylates AS160 on Thr^{642} site. AMPK-dependent phosphorylation of TBC1D1 on Ser^{237} may play a role in translocating contraction-responsive pool of GLUT4 (associated with TBC1D1) to the cell-surface membranes. In vitro contraction stimulates AS160 Thr^{642} phosphorylation in Akt-dependent manner (on insulin-responsive pool of GLUT4). However, AS160 Thr^{642} phosphorylation via contraction is not sufficient for this pool of GLUT4 to become translocated to the cell-surface membranes. We hypothesize that these GLUT4 vesicles which are associated with $pThr^{642}$AS160 are more susceptible to being fully translocated to cell surface membranes by subsequent insulin stimulation. B) The reversal of the insulin-independent increase in glucose transport was found with dephosphorylation of TBC1D1 on Ser^{237} at 3 hours post-exercise (internalized GLUT4 vesicles may become reassociated with unphosphorylated TBC1D1). In contrast, AS160 Thr^{642} phosphorylation remains elevated. We speculate that the persistently elevated AS160 Thr^{642} phosphorylation that was found at 3 and 27 h post-exercise plays a role in post-exercise increase in insulin-stimulated glucose transport.

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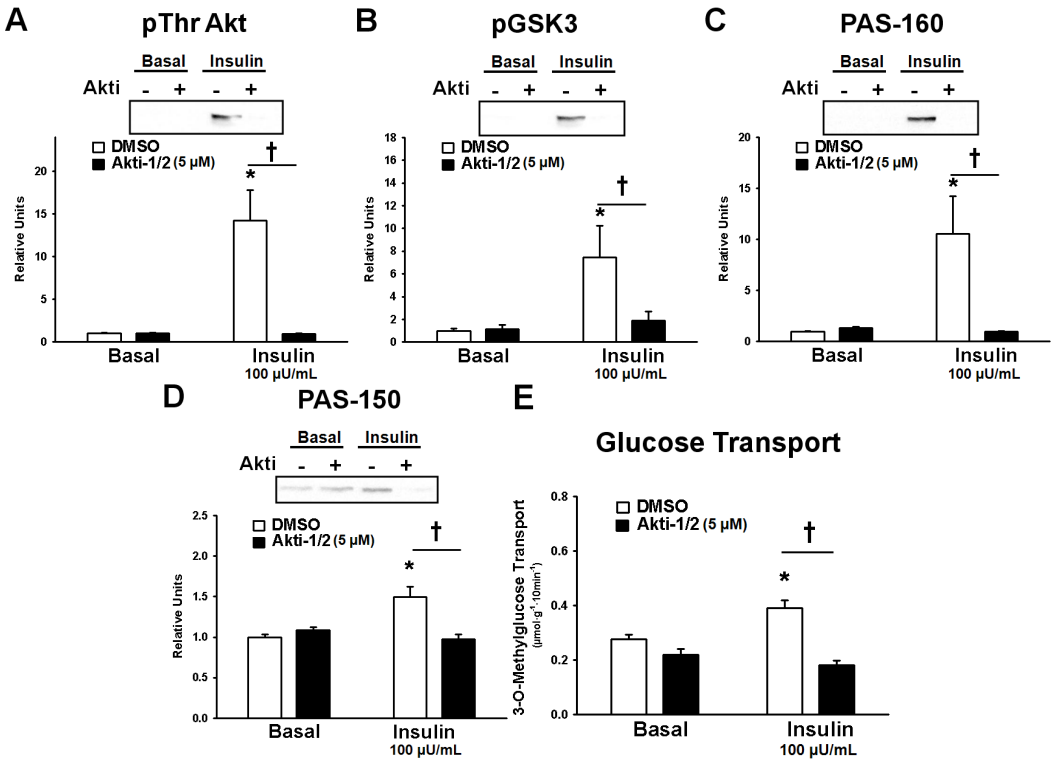
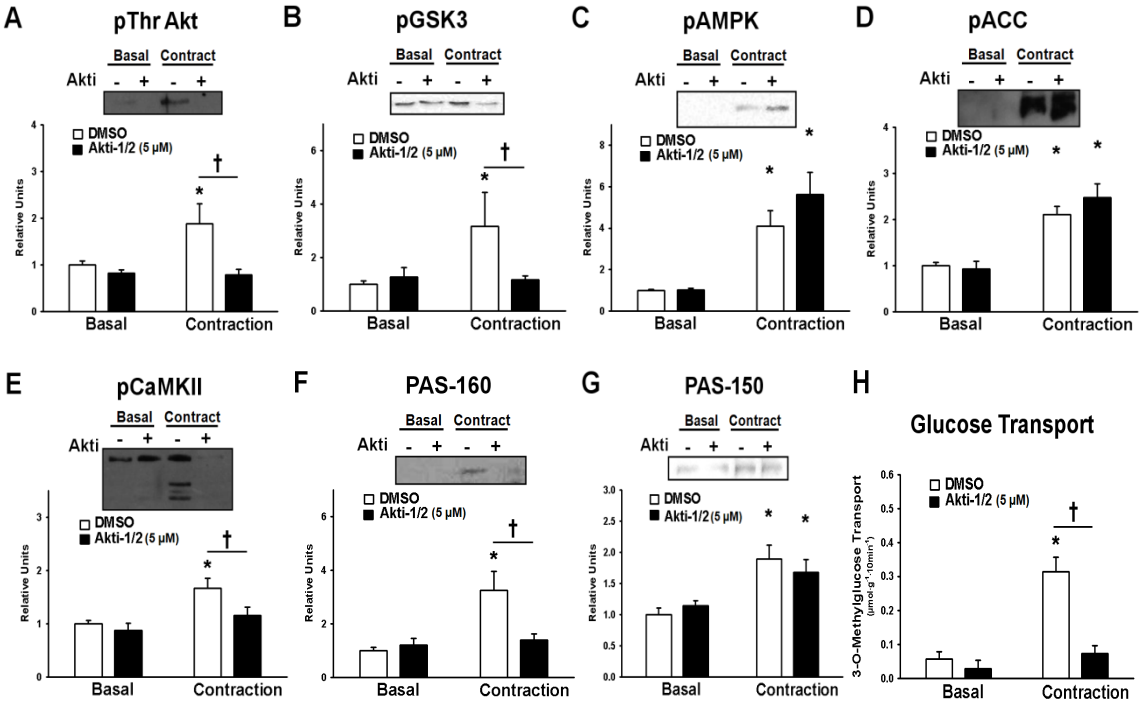
APPENDICES

Appendices A to D include a summary of some of the unpublished data collected at the University of Michigan that was not included in Studies 1 to 4 of this dissertation.



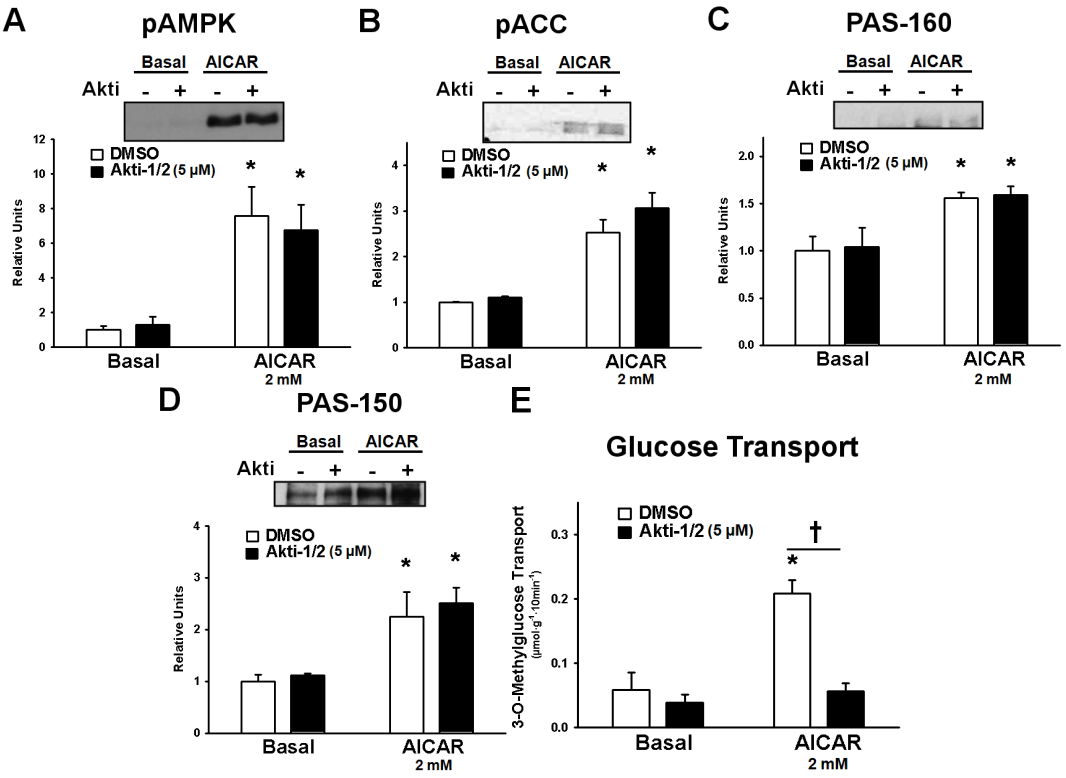
Appendix A

Effects of Akti-1/2 on insulin-stimulated phosphorylation of Akt^{Thr308}, GSK3$^{Ser21/9}$, PAS-160, PAS-150 and glucose transport. (A) pAkt^{Thr308}, (B) pGSK3$^{Ser21/9}$, (C) PAS-160, (D) PAS-150 and (E) glucose transport. For all incubation steps, media solution contents and conditions were identical to those in Study 2. Paired isolated rat epitrochlearis muscles were incubated with or without 5 μM of Akti-1/2 (Akt inhibitor, Sigma-Aldrich, A6730) for 60 min. Muscles were then either incubated in identical media (Basal) or in solution that contained 100 μU/mL of insulin for 20 min, freeze clamped immediately and used for immunoblotting or for 3-MG transport measurement. Data are means ± S.E., n = 6-8/group. Post hoc analysis: *, $P < 0.05$ (effect of insulin); †, $P < 0.05$ (effect of Akti-1/2). Open bars = DMSO; filled bars = Akti-1/2.



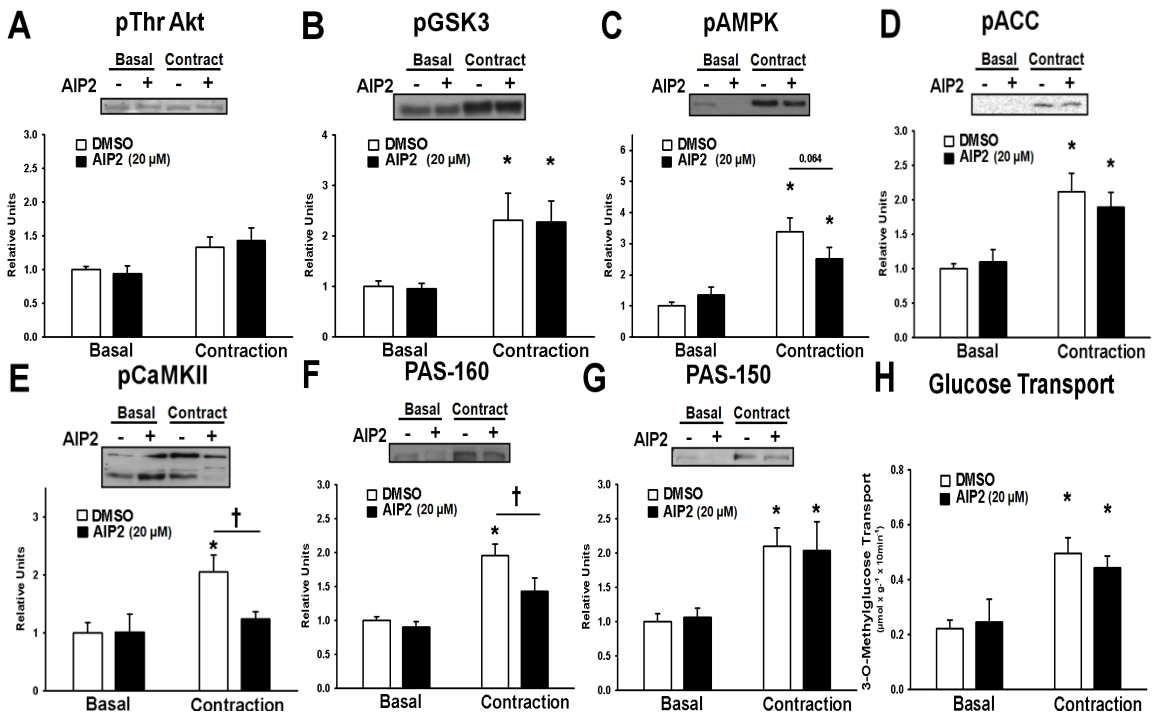
Appendix B

Effects of Akti-1/2 on contraction-stimulated phosphorylation of Akt^{Thr308}, GSK3$^{Ser21/9}$, AMPK^{Thr172}, ACC^{Ser79}, CaMKII^{Thr286}, PAS-160, PAS-150 and glucose transport. (A) pAkt^{Thr308}, (B) pGSK3$^{Ser21/9}$, (C) pAMPK^{Thr172}, (D) pACC^{Ser79}, (E) pCaMKII^{Thr286}, (F) PAS-160, (G) PAS-150, (H) glucose transport. For all incubation steps, media solution contents and conditions were identical to those in Study 2. Paired isolated rat epitrochlearis muscles were incubated with or without 5 μM of Akti-1/2 (Akt inhibitor, Sigma-Aldrich, A6730) for 60 min. Muscles were then either incubated in identical media (Basal) or were stimulated to contract (Contract, 2 ms twitch, 2 Hz) for 20 min, freeze clamped immediately and used for immunoblotting or for 3-MG transport measurement. Akti-1/2 did not affect peak force or total force production. Data are means ± S.E., n = 6-8/group. Post hoc analysis: *, $P < 0.05$ (effect of contraction); †, $P < 0.05$ (effect of Akti-1/2). Open bars = DMSO; filled bars = Akti-1/2.



Appendix C

Effects of Akti-1/2 on AICAR-stimulated phosphorylation of AMPK^{Thr172}, ACC^{Ser79}, PAS-160, PAS-150 and glucose transport. (A) pAMPK^{Thr172}, (B) pACC^{Ser79}, (C) PAS-160, (D) PAS-150, (E) glucose transport. For all incubation steps, media solution contents and conditions were identical to those in Study 2. Paired isolated rat epitrochlearis muscles were incubated with or without 5 μM of Akti-1/2 (Akt inhibitor, Sigma-Aldrich, A6730) for 60 min. Muscles were then either incubated in identical media (Basal) or in solution that contained 2 mM of 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR, AMPK activator) for 40 min, freeze clamped immediately and used for immunoblotting or for 3-MG transport measurement. Data are means ± S.E., n = 6/group. Post hoc analysis: *, $P < 0.05$ (effect of contraction); †, $P < 0.05$ (effect of Akti-1/2). Open bars = DMSO; filled bars = Akti-1/2.



Appendix D

Effects of AIP2 on contraction-stimulated phosphorylation of Akt^{Thr308}, GSK3$^{Ser21/9}$, AMPK^{Thr172}, ACC^{Ser79}, CaMKII^{Thr286}, PAS-160, PAS-150 and glucose transport. (A) pAkt^{Thr308}, (B) pGSK3$^{Ser21/9}$, (C) pAMPK^{Thr172}, (D) pACC^{Ser79}, (E) pCaMKII^{Thr286}, (F) PAS-160, (G) PAS-150, (H) glucose transport. For all incubation steps, media solution contents and conditions were identical to those in Study 2. Paired isolated rat epitrochlearis muscles were incubated with or without 20 μM of AIP2 (CaMKII inhibitor, autocamtide-2 related inhibitory peptide II, EMD Biosciences, no. 189484) for 120 min. Muscles were then either incubated in identical media (Basal) or were stimulated to contract (Contract, 2 ms twitch, 2 Hz) for 20 min, freeze clamped immediately and used for immunoblotting or for 3-MG transport measurement. AIP2 did not affect peak force or total force production. Data are means ± S.E., n = 6-8/group. Post hoc analysis: *, $P < 0.05$ (effect of contraction); †, $P < 0.05$ (effect of AIP2). Open bars = DMSO; filled bars = AIP2.