Mechanisms for Calorie Restriction Effects on Insulin-stimulated Glucose Uptake by Rat Skeletal Muscle

by

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Dedicated to the memory of LaMont Toliver, 1963-2012

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ABSTRACT

Hallmarks of calorie restriction (CR; consuming ~60% of ad libitum, AL, intake) include greater whole body insulin sensitivity and insulin-stimulated glucose uptake (ISGU) by skeletal muscle. This thesis aimed to extend knowledge on mechanisms for CR-effects on ISGU by elucidating: 1) CR-effects on insulin signaling and muscle ISGU in old age, 2) mechanisms for CR-enhanced Akt phosphorylation (pAkt), and 3) in vivo signaling and ISGU in multiple muscles of varying fiber types. The results demonstrated that 24-month-old CR versus AL rats had greater ISGU in isolated epitrochlearis and soleus muscles with greater pAkt and phosphorylation of Akt-substrate filamin C, but not greater phosphorylation of Akt-substrates AS160 or TBC1D1 or insulin receptor tyrosine phosphorylation. Greater pAkt in isolated soleus from 9-month-old CR versus AL rats was not attributable to greater activation of signaling steps proximal to Akt (insulin receptor phosphorylation, insulin receptor substrate-1 tyrosine phosphorylation or greater insulin receptor substrate-1-phosphatidylinositol-3-kinase activity at 5, 15 or 50 minutes insulin exposure). Analysis of six muscles from 9-month-old rats with in vivo insulin exposure revealed that CR versus AL rats had greater pAkt in each of four predominantly Type II muscles (epitrochlearis, gastrocnemius, tibialis anterior and plantaris) and one of two predominantly Type I muscles (soleus, but not adductor longus) with greater ISGU for three of four predominantly Type II muscles (not plantaris) and neither predominantly Type I muscle and no diet-effect on insulin receptor tyrosine phosphorylation for any

muscle. In conclusion: 1) the CR-related increase in insulin-stimulated pAkt regardless of age or experimental model (in vivo or ex vivo) in multiple muscles suggests that Akt may be necessary, but not sufficient for a CR-related increase in muscle ISGU; 2) greater pAkt for CR versus AL rats was not attributable to detectable diet-related differences in insulin signaling steps proximal to Akt suggesting CR-effects on pAkt may rely on other mechanisms such as modulation by regulatory proteins that bind to Akt; and 3) the similarity for CR-related effects on ex vivo and in vivo ISGU for the epitrochlearis, but not the soleus, suggested a muscle-specific difference in susceptibility to the influence of systemic factors.

CHAPTER I

INTRODUCTION

Insulin resistance is a condition in which a physiological amount of insulin leads to subnormal glucose disposal into insulin target tissues and inadequate suppression of hepatic glucose production. It is a primary defect in type 2 diabetes (T2DM) and is also an independent risk factor for several chronic diseases that increase in prevalence with advancing age (1). In particular, skeletal muscle can clear as much as 85% of the blood glucose load, therefore is a very significant peripheral tissue for preventing the accumulation of glucose into the blood (2). It would be tremendously valuable to elucidate the mechanisms of insulin stimulated glucose transport and to develop meaningful therapies that may serve as interventions that increase insulin sensitivity particularly in aging models.

Calorie restriction without malnutrition (CR; consuming ~60 to 75% of ad libitum food intake) is characterized by multiple adaptations that can lead to improved function and health in many species. One of the hallmarks of CR is improved insulin sensitivity which is largely secondary to increased insulin-stimulated glucose uptake by skeletal muscle. The greater glucose uptake by insulin-stimulated muscle is attributable to greater

recruitment of GLUT4 to cell surface membranes (3). Many studies have found that CR does not elevate skeletal muscle GLUT4 protein abundance, which suggests that the CR effect on glucose transport depends on improved function of insulin signaling and/or GLUT4 vesicle trafficking proteins (4-7). Insulin binds to its receptor to stimulate tyrosine autophosphorylation to phosphorylate insulin receptor substrates (IRS). IRS1 is the predominant isoform in skeletal muscle, and tyrosine phosphorylated-IRS1 binds phosphatidylinositol 3-kinase (PI3K), which is essential for insulin to induce GLUT4 translocation. A key post-PI3K activator of insulin-stimulated glucose transport is the Ser/Thr kinase known as Akt. A consistent effect of CR is a substantial increase in the insulin-stimulated Akt phosphorylation, on two important sites (Thr308 and Ser473) important for insulin mediated glucose uptake (8-13). Mammals express three Akt isoforms that have distinct, but overlapping functions, and Akt1 and Akt2 are abundantly expressed by skeletal muscle. Akt2 has been identified as the isoform that is crucial for insulin-mediated GLUT4 translocation and glucose uptake (14-17). Insulin also causes a protein named Akt substrate of 160 kDa (AS160) to become phosphorylated on multiple Akt-phosphomotifs (18). Two sites (Thr642 and Ser588) were shown to be crucial for a large portion, but not all, of insulin-mediated GLUT4 translocation to cell surface membranes (19).

This dissertation aimed to extend knowledge on mechanisms for CR-effects on insulin-stimulated glucose uptake by elucidating: 1) CR-effects on insulin signaling and muscle insulin-stimulated glucose uptake in old age, 2) mechanisms for CR-enhanced Akt phosphorylation, and 3) in vivo signaling and insulin-stimulated glucose uptake in multiple muscles of varying fiber types.

Study 1: Calorie Restriction Enhances Insulin-stimulated Glucose Uptake and Akt Phosphorylation in Both Fast-twitch and Slow-twitch Skeletal Muscle of 24 Month-old Rats

Earlier studies have assessed the influence of CR on various aspects of the insulin signaling pathway that regulates GLUT4 vesicles in skeletal muscle in adult animals (3, 8-10, 20-25). In contrast to the relatively extensive amount of research on the mechanisms for the CR-induced elevation in insulin-stimulated glucose uptake in muscles from adult animals, little is known about CR effects on insulin signaling in muscle from old rats. A few studies have evaluated the influence of CR on insulin receptor function in skeletal muscle of old rats (6, 26, 27), but these studies did not determine if there were fiber type differences. The effects of CR on Akt or AS160 phosphorylation in insulin-stimulated muscle from old rats, regardless of fiber type, have not been reported. Furthermore, the influence of CR on GLUT4 abundance in the epitrochlearis and soleus of old rats is unknown.

Study 2: Comparison of Ad Libitum Fed and Calorie Restricted Rats for the Time Course of Insulin's Activation of Signaling Steps Required for Greater Glucose Transport by Isolated Skeletal Muscle

Most (28-30), but not all (6) of the published studies that have evaluated CR effects on skeletal muscle insulin receptor (IR) function at submaximally effective insulin levels have not found diet-induced differences. In a previous study (24), there were no significant diet effects on IR tyrosine phosphorylation or IRS1-PI3K activity with a physiologic insulin concentration. These results suggest elevation in Akt phosphorylation

with CR was not attributable to enhancement in insulin receptor or IRS1-PI3K activity (13). The goal of this project is to determine the time course of insulin signaling with CR versus. AL in response to a physiological insulin dose and determine if these observations may be attributable to reversal of transient CR effect on proximal steps of insulin signaling that may have reversed by the time point at which previous studies have evaluated insulin signaling (typically after ~50 minutes of insulin exposure)**.**

Study 3: In Vivo Assessment of the Effects of Calorie Restriction on Insulin-stimulated Glucose Uptake and the Insulin Signaling Pathway in Multiple Skeletal Muscles

Compared to the relatively detailed information about CR effects on insulin signaling in muscle ex vivo, relatively few studies have focused on insulin signaling in rodent muscle of CR rats exposed to insulin in vivo. A major gap in current knowledge is the lack of any published data on the effect of in vivo administration of a physiologic insulin dose on insulin signaling in skeletal muscle from CR compared to AL rats. The purpose of this study was to evaluate the mechanisms of in vivo insulin-stimulated glucose uptake and phosphorylation of key insulin regulated proteins in multiple skeletal muscles from adult ad libitum fed and CR (consuming 65% of ad libitum intake) 9mo-old Fisher 344 X Brown Norway rats under euglycemic-hyperinsulinemic clamp conditions.

CHAPTER II

REVIEW OF LITERATURE

Significance of Calorie Restriction

Multiple lines of evidence indicate that calorie restriction (CR; consuming ~60-75% of ad libitum intake) has beneficial effects on health span and extends lifespan across various species, including worms, fish, flies, mice, and rats (31-33). Although knowledge of CR effects on lifespan in humans is not entirely clear due to difficulty in conducting the proper scientific studies, there is an on-going 20-year longitudinal study on CR effects in non-human primates that demonstrates some important benefits of CR, including delayed mortality and reduced incidence of diabetes, cancer, and cardiovascular disease (34). Older rhesus monkeys undergoing long-term CR exhibit benefits on skeletal muscle, including attenuation of sarcopenia (35) and increased insulin sensitivity (36) thereby delaying common age-related declines in skeletal muscle function and insulin resistance.

Increased insulin sensitivity and reduced plasma insulin levels are hallmarks of CR in multiple species including mice (8, 37), rats (24, 38), monkeys (39), and humans (40-42). Insulin resistance is defined as a subnormal response to a normal dose of

insulin. It is associated with several other chronic diseases including type 2 diabetes, atherosclerosis, hypertension, malignancy, and cognitive dysfunction (2, 43, 44). Thus, CR serves as an intervention that may help alleviate or prevent prevalent age-related pathologies associated with insulin resistance.

Significance of Skeletal Muscle Glucose Transport

Insulin resistance is a precursor for the progression to type 2 diabetes mellitus (T2DM). Twenty years ago T2DM was primarily diagnosed as a disease of adulthood. However, with increased incidence, T2DM now is estimated in the world to account for as much as 45% of diabetes diagnosed in children and as much as 95% of all diabetic cases in the United States as of 2007 (45, 46). In 2000, 8.8% of U.S. adults at least 20 years of age were diagnosed with diabetes and this estimate is expected to rise to 11.2% of the population by 2030 (46). The economic burden and health consequences of T2DM are therefore expected to increase significantly within the next two decades.

Glucose transport in skeletal muscle occurs by facilitated diffusion mediated by glucose transporter proteins, with the GLUT4 transporter responsible for insulin-stimulated glucose transport (47-50). Glucose transport is a rate controlling step for glucose metabolism in skeletal muscle cells (51). Insulin induces increased glucose uptake into insulin-sensitive cells (including skeletal muscle and adipocytes), and thus regulates blood glucose levels especially after feeding (27). In particular, skeletal muscle can dispose of as much as 85% of insulin-induced clearance of the blood glucose load (10). Chronic hyperglycemia is a diagnostic sign of diabetes mellitus which is the leading cause of blindness due to retinopathy, end stage renal disease, and lower

extremity amputations. In addition to hyperglycemia, compensatory hyperinsulinemia ensues further contributing to disease (43, 44, 52). High blood insulin concentrations result from the increased production of insulin from the pancreas in an attempt to overcome insulin resistance in peripheral tissues, i.e. skeletal muscle. Given that skeletal muscle is the tissue that accounts for the greatest amount of whole body glucose disposal (2), elucidating interventions that increase insulin-mediated glucose uptake by skeletal muscle (38, 53, 54) is critically important in the context of an obesity and the T2DM epidemic

It has been well-established that insulin stimulates GLUT4 translocation. A tremendous amount of research has been performed to elucidate the specific mechanisms that regulate insulin-stimulated GLUT4 translocation and glucose transport in skeletal muscle with the goal of developing meaningful therapies that may serve as interventions for hyperinsulinemia and hyperglycemia. A full understanding of skeletal muscle glucose uptake regulation via the insulin signaling pathway is important to maximize targets for interventions that aim to reverse defects in glucose metabolism.

The Insulin Signaling Pathway

The insulin receptor is a heterotetrameric protein consisting of two alpha subunits and two beta subunits. The alpha subunits are located on the extracellular surface of the plasma membrane and contain the insulin binding site. The beta subunits span the plasma membrane and are involved with intracellular signaling (55). Insulin action begins by binding to the alpha subunit of the insulin receptor on the cell surface (56) and triggers autophosphorylation of tyrosine residues on the beta subunit of the insulin receptor (57).

Mutations of insulin receptor tyrosine residues 1162 and 1163 reduce insulin-stimulated autophosphorylation of the insulin receptor, receptor tyrosine kinase activity and insulin-stimulated uptake of 2-deoxyglucose (58). Insulin receptor autophosphorylation enables the recruitment and phosphorylation of the intracellular adaptor proteins called insulin receptor substrates (IRS). IRS-1 is the predominant isoform in skeletal muscle, and skeletal muscle IRS-1 is important for normal glucose homeostasis (59, 60). IRS-1 associates with and activates the phosphoinositol 3 kinase (PI3K) enzyme. Activated PI3K phosphorylates the 3rd position on the inositol ring of phosphoinositides (61) catalyzing the formation of phosphatyidyinositol-3,4-biphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3). PIP3 recruits serine kinases (including PDK1 and Akt) to the plasma membrane which are important for insulin transduction (62).

Akt, a serine kinase downstream of the PI3K, regulates many diverse functions in the cell including glucose transport, protein synthesis, glycogen synthesis, cellular growth and development. Of the three isoforms of Akt, Akt 1 and Akt 2 are the predominant Akt isoforms expressed in skeletal muscle (63-66). Upstream phosphoinositide-dependent kinases-1 (PDK1) and mammalian target of rapamycin complex-2 (mTORC2) activate Akt by phosphorylation on T308 and S473 residues, respectively. Through knockout mice and cell culture mutation analysis it has been demonstrated that Akt1 is important for growth and development while Akt2 is primarily responsible for regulating glucose metabolism (67, 68). Further, Ng et al. reported that Akt2 is sufficient to stimulate GLUT4 translocation (69). Akt phosphorylates various protein substrates on serine or

threonine residues in the RXXRXXS/T consensus motif (70). Therefore substrates containing this motif are candidates that may link Akt to glucose transport.

A protein named Akt substrate of 160kDa (AS160) was discovered to be insulin responsive (18) and involved in GLUT4 translocation (19). AS160 was characterized in 3T3-L1 adipocytes and, in addition to being insulin responsive, was determined to be located downstream of the PI3K-Akt pathway. It is the most distal insulin signaling protein to date that has been clearly linked to GLUT4 translocation. AS160 contains a Rab GTPase activating protein (GAP) domain suggesting a possible role in insulin dependent vesicular trafficking (71). It was revealed that AS160 contained 5 potential Akt target phosphomotiffs (RXXRXXS/T). Kane et al. (18) demonstrated that AS160 is a substrate of Akt by incubating AS160 with recombinant Akt1 in vitro. To demonstrate the link between AS160 and GLUT4 translocation, Sano et al. (19) produced mutations on one key Akt phosphomotif (Thr642) on AS160 and found a significant decrease in insulin stimulated GLUT4 translocation. Although these initial findings characterized AS160's role as a regulator of insulin-stimulated glucose transport in 3T3-L1 cells, it was also recognized that AS160 was expressed by skeletal muscle. Studies by Arias et al. (72) and Bruss et al. (73) revealed that insulin increases AS160 phosphorylation in rat skeletal muscle. Insulin-stimulated AS160 phosphorylation was eliminated by the PI3K inhibitor wortmannin indicating that the increase in AS160 phosphorylation in muscle is PI3K dependent. Furthermore, in skeletal muscle from Akt2 knockout mice, an intraperitoneal injection of insulin did not increase AS160 phosphorylation suggesting that Akt2 is the key isoform that phosphorylates AS160 with insulin stimulation (74). AS160 was also shown to be important for glucose uptake in skeletal muscle. Overexpression of AS160

that was mutated on 4 key insulin-responsive Akt phosphomotifs (4P mutant) inhibited ~50% of the insulin-stimulated increase in glucose uptake in skeletal muscle. The results in skeletal muscle support the idea that AS160 is essential for the full effect of insulin on glucose uptake in skeletal muscle.

Although AS160-dependent mechanisms are important for a substantial portion of insulin-mediated glucose uptake, it is not sufficient for the full effect. TBC1D1 is a recently discovered paralog of AS160. TBC1D1 also has a GAP domain, regulating Rab activity, and it can regulate GLUT4 translocation in 3T3- L1 cells. Chen et al.(75) demonstrated in a cell free assay that Akt can phosphorylate TBC1D1 on several sites. TBC1D1 can also modulate glucose uptake in skeletal muscle. Therefore, TBC1D1 could function as an Akt-dependent substrate important for glucose transport regulation in skeletal muscle.

Philip Cohen's group identified the cytoskeleton protein filamin C (FLNc) as an Akt substrate. FLNc was shown to be phosphorylated on S2213 with insulin stimulation and this phosphorylation was prevented by the presence of the specific PI 3-kinase inhibitor wortmannin in C2C12 myoblasts (76). Filamin C is highly expressed in skeletal muscle and is reported to stabilize actin filament networks on cell membranes (77). Insulin signaling molecules and translocation of GLUT4 transporter vesicles to the cell membrane has been shown to rely on the integrity and maintenance of actin filaments (78, 79). Furthermore, CR increased filamin C phosphorylation in the rat epitrochlearis muscle stimulated with physiological insulin (Sharma et al. unpublished).

Relationship between Skeletal Muscle Fiber Type and Insulin-Stimulated Glucose Transport

Skeletal muscle is a heterogeneous tissue and specific muscles can vary greatly in their fiber type composition. The gold standard method for categorizing fiber types is identifying each skeletal muscle fiber by its myosin heavy chain (MHC) expression. In adult rat skeletal muscle, type I, IIA, IIB, and IIX are the myosin isoforms expressed. This heterogeneity can be accompanied by different metabolic characteristics (i.e. insulin-stimulated glucose uptake) for a particular skeletal muscle. Insulin-stimulated glucose uptake is regulated by the GLUT4 transporter and GLUT4 abundance has been shown to vary within different isolated rat skeletal muscles (80, 81). When this variability was correlated with the fiber type, studies suggest that GLUT4 abundance is greater for oxidative (type I) fibers or the fast-oxidative (type IIA) compared to fast-glycolytic fibers (IIB) (81, 82). The results from these studies demonstrate the importance of considering skeletal muscle fiber type make-up and its capacity for insulin-stimulated glucose transport. Rat skeletal muscle composed of primarily of type I oxidative fibers (soleus) and type IIA fibers (flexor digitorum brevis) were both shown to have at least two fold higher insulin stimulated glucose uptake compared to skeletal muscle with a greater proportion of fast-glycolytic fibers(80). Furthermore, MacKrell et al. (83) studied insulin-stimulated glucose uptake in isolated skeletal muscle single fibers from rats and found that fast-oxidative fibers (type IIA) have significantly higher glucose uptake rates compared to fast-glycolytic fibers (type IIB). The data together suggest that type I and type IIA fibers have more similar profiles when compared to type IIB and type IIX fibers with respect to insulin stimulated glucose transport. A full understanding of

the mechanisms of insulin-stimulated glucose transport in skeletal muscle requires knowledge on multiple skeletal muscle tissue as each skeletal muscle is composed of a unique metabolic profile based on its own fiber type composition.

Effects of Aging on Skeletal Muscle Insulin Signaling and Glucose Transport

Identifying defects in skeletal muscle insulin signaling which are responsible for the inability of the muscle cell to properly dispose of blood glucose are important in understanding both primary and secondary aging as causes of insulin resistance. There is progressive insulin resistance, even in "healthy" individuals who will not progress to the development of Type 2 diabetes as they age. However, very little is understood about the specific mechanisms that cause these primary age-related changes in muscle glucose metabolism. Previous studies have revealed a subtle yet significant decrement in glucose uptake in both skeletal muscle in vivo and isolated skeletal muscle (84). The euglycemic-hyperinsulinemic clamp technique is the gold standard for assessing whole body in vivo insulin sensitivity. Results from in vivo experiments include the direct influences of various factors absent from ex vivo experiments, including: blood flow, neural inputs, blood hormones and cytokines, in addition to the intrinsic characteristic of the muscle. Previous studies using this technique assessed whole body insulin resistance and observed that old rats (20 to 24 month old rats) compared to adult (6 to 10 month old rats) have an age-related insulin resistance in peripheral tissues which includes skeletal muscle. The euglycemic-hyperinsulinemic clamp in combination with use of an injectable radiolabeled 2DG tracer allows measurement of 2DG uptake in individual skeletal muscles. One study using this procedure found an age-related decrement in

insulin sensitivity in the predominantly slow twitch soleus muscle, but not in the predominantly fast twitch quadriceps femoris. This result suggests that not all skeletal muscles become resistant with age in the face of whole body age-related insulin resistance. In order to more closely examine this phenomenon intrinsic to the skeletal muscle, Sharma et al.(84) evaluated glucose uptake by both the isolated soleus and epitrochlearis muscles from 9 month old and 25 month old rats ex vivo. Advantages of ex vivo preparations include more experimental control and measurements are separated from sustained systemic effects. In the predominantly slow-twitch soleus muscle there was age-related insulin resistance for glucose uptake, however for the predominantly fast-twitch epitrochlearis insulin stimulated glucose uptake there was not a significant difference between the 9 month and 25 month old rats. The extent of age-related resistance therefore is not uniform in all muscles. Aging has variable effects on insulin-stimulated glucose transport within muscles that vary in their metabolic profiles. The GLUT4 transporter carries glucose into the muscle cell from the bloodstream in response to insulin. Despite the differences in glucose transport with age in skeletal muscle, research studies show that there are not differences in total GLUT4 content in adult rats versus old rats. Thus age-related insulin resistance on each individual skeletal muscle may be explained by an aging effect on the insulin signaling pathway which regulates GLUT4 trafficking and/or defects in the GLUT4 trafficking process.

The study by Sharma et al. was also one of the seminal papers to evaluate age-related resistance on key insulin signaling proteins in 9 versus 24 month rats. In isolated epitrochlearis and soleus muscles that were incubated ex vivo with 1.2 nM (physiologic) or 30 nM (supraphysiologic) insulin, insulin signaling (Akt and AS160) was examined in

adult versus old rats. In the epitrochelaris, there was no decrease reported in the major skeletal muscle isoforms pAkt1 T308 and pAkt2 T308 in the old versus adult group with 30nM of insulin. There were similarly no age-related changes seen in either of the isoforms at the T308 site with 1.2nM of insulin. At Akt S473 (the other important Akt insulin-stimulated phosphorylation site) in the epitrochlearis there were no age related changes at basal or physiological insulin, but with a supraphysiological insulin level there was an age-related decrement. In the soleus, there was an age related decrement in pAkt2 T308 at 30nM insulin and a trend to decrease with 1.2nM insulin. In the soleus there was a trend (P=0.07) for a decrease in glucose uptake in 24 versus 9 month old rats with 1.2nM insulin, and a significantly reduced glucose uptake with 30 nM insulin. It seems likely that Akt2 is related to the decrease in insulin stimulated glucose uptake with age. When the Akt substrate, AS160 was evaluated at the critical T642 site there was no apparent age-related decrease in AS160 phosphorylation at any insulin concentration. This result seemed to suggest that age-related resistance occurs through an AS160 independent mechanism through other Akt substrates. However, AS160 is regulated on multiple insulin-regulated phosphorylation sites, and it can be regulated by binding to 14-3-3 proteins. Therefore AS160 cannot be ruled out completely. Further studies in aging muscle are required to fully elucidate the mechanisms that account for glucose transport and insulin signaling particular in the face of primary age-related resistance.

Effect of CR on Adult and Old Rat Skeletal Muscle Insulin Signaling and Glucose Transport

Calorie restriction is well known to enhance insulin-stimulated glucose transport in skeletal muscle. Previous studies have documented the CR effect on insulin stimulated glucose uptake in adult rats (3, 10, 21, 25, 85-87) and mice (8, 37) in both epitrochlearis and soleus muscles. In 23 month-old rats, CR was to shown to increase insulin stimulated glucose uptake in the epitrochlearis muscle. Dean et al. (3) demonstrated that this effect is attributable to a proportional CR-mediated increase in the cell surface GLUT4 at the plasma membrane in rat skeletal muscle. The effect of CR on insulin-stimulated glucose transport can occur in the absence of increased total GLUT4 protein abundance (88). These results suggest that the mechanism for the CR effect on skeletal muscle glucose transport is related to the specific enhancement of the insulin signaling pathway leading to increase GLUT4 recruitment to the plasma membrane.

A few studies have evaluated the effect of CR on the insulin signaling pathway in skeletal muscle, but the mechanisms that account for the CR enhancement of insulin-stimulated glucose uptake are still not entirely clear. In adult rodents, most studies that have evaluated the insulin receptor activation with physiological doses of insulin do not report a CR effect (24, 29). Roughly half of the studies that looked at the insulin receptor with a supraphysiological dose of insulin found significant changes with the remaining studies not detecting altered insulin receptor function with CR (6, 7, 23, 27, 29, 89, 90). In old rats, a few studies have evaluated the effect of CR on insulin receptor function in skeletal muscle by using an insulin injection. CR does not seem to alter insulin receptor function in skeletal muscle (24) unless a supraphysiological dose of insulin is present (26, 27).

Post-insulin receptor signaling has also been shown to increase in some, but not all studies using a supraphysiological dose of insulin (3, 7, 10, 23, 25, 89, 90). With a physiological does of insulin several studies do not report a significant CR versus AL change in either IRS-1 associated PI3K signaling or IRS-1 tyrosine phosphorylation in rat skeletal muscle (24). However, other studies have reported that muscles from mice (7) and monkeys (6) reported that CR resulted in greater IRS-1-PI3K activity. Taken together, these results suggest that CR may affect the insulin signaling pathway downstream of PI3-kinase.

Akt and atypical PKC are key insulin signaling proteins downstream of PI3 kinase that have been implicated in the regulation of insulin stimulated glucose uptake in skeletal muscle. Insulin-stimulated aPKC was reduced in skeletal muscle from obese non-diabetic and diabetic rodent skeletal muscle (91, 92). However, Sharma et al. (24) studied aPKC in isolated rat skeletal muscle (epitrochlearis and soleus) from adult rats and found that aPKC activity did not differ with CR versus AL. Insulin-stimulated Akt phosphorylation has been the most consistent and striking result with CR versus AL in adult rats. In numerous studies Akt phosphorylation increases at two key sites (T308 and S473) in skeletal muscle undergoing CR (8-10, 24). This effect is seen with both supraphysiologic and physiological levels of insulin. Cho et al. (68) showed that Akt2 is the critical isoform for regulating most of the insulin-stimulated glucose transport in skeletal muscle. McCurdy et al. (9) revealed that Akt2 at both T308 and S473 were increased for CR versus. AL with rodent skeletal muscle stimulated with a submaximally effective level of insulin. The effects of CR on Akt phosphorylation in insulin-stimulated muscle in old rats in not known. These previous reports have demonstrated that Akt is

likely important for the CR enhanced insulin-mediated GLUT4 translocation and glucose uptake in skeletal muscle. However, further studies elucidating the specific Akt substrates that link Akt signaling to the greater insulin-induced GLUT4 translocation and glucose transport in response to CR are important.

CR has been shown to increase insulin-stimulated phosphorylation of AS160 in skeletal muscle in adult rats. Sharma et al. (24) evaluated AS160 phosphorylation in the epitrochlearis and found that AS160 phosphorylation (T642 and S588) was significantly greater in the CR than the AL group with a physiological dose of insulin. In the soleus, there was no significant dietary effect on AS160 phosphorylation with either a physiologic or supraphysiologic insulin concentration. The CR effect on AS160 phosphorylation in insulin stimulated skeletal muscle from old rats has not been reported.

Role of CR on In Vivo Insulin Signaling and Glucose Transport

The euglycemic-hyperinsulinemic clamp is considered the gold standard for assessing in vivo insulin sensitivity at the whole body level. In vivo skeletal muscle glucose uptake can be specifically evaluated by applying the clamp technique together with infusion of radiolabeled 2-deoxyglucose to rodents. There is limited literature regarding the effect of CR on insulin signaling and glucose uptake in rodents in skeletal muscles in vivo. Wetter et al. (22) utilized an in vivo radioactive 2DG tracer method to assess the CR effects on in vivo glucose uptake by various skeletal muscles under endogenous insulin levels (i.e., without insulin infusion) in adult rats. Plasma insulin was reported to be significantly lower in CR versus AL rats, consistent with increased whole body insulin sensitivity. The skeletal muscle of CR versus AL rats had at least equal or

higher in vivo glucose uptake values despite having lower insulin levels. These results suggest that CR versus AL enhances in vivo insulin sensitivity and enhances tissue specific glucose uptake in skeletal muscle. In a more recent study, the euglycemic-hyperinsulinemic clamp was used to study in vivo glucose metabolism with CR in both adult and old rats (93). Wistar rats at 8 month and 24 months of age were used to determine CR's effect on in vivo insulin sensitivity in skeletal muscle as function of age. With a physiologically-effective dose of insulin it was reported that CR significantly increased insulin-stimulated glucose uptake in the soleus and quadriceps at 24 months. At 8 months of age there was a statistically non-significant trend for increased glucose uptake in the soleus with CR, but in the quadriceps there was no observed difference between CR versus AL rats. In addition the average insulin level during the clamp procedure in the 8 month rats with CR was significantly lower versus AL animals.

Although there is a large body of evidence of the beneficial effects of CR, little is known about the diversity of CR effects in different skeletal muscles. Therefore, it would be valuable to perform an assessment of the effects on insulin signaling and glucose uptake with CR in multiple skeletal muscles under controlled physiologic experimental conditions. Apparently no published studies have assessed the influence of long-term (several months) of moderate CR on insulin signaling in rat skeletal muscle collected in vivo with a physiologic insulin concentration.

Rationale for Models Used in this Research

Rats were used for the experiments in this dissertation research. Rats are a useful model for humans with regard to the influence of age on insulin-stimulated glucose

uptake. Like humans, rats have a modest decline in glucose disposal from young to old age (~15-35%) (94, 95). Calorie restriction (CR) is used in this thesis as a dietary intervention that has been shown to increase insulin sensitivity and therefore is a useful model to explore as an intervention to reverse insulin resistance and associated pathology. Past research exploring the CR effects on insulin signaling and glucose uptake using rat skeletal muscle has been well established including but not limited to: increase cell surface GLUT4 with CR (3), increase glucose uptake with CR (24, 37), insulin signaling with CR (6, 9, 10, 23-25, 96). These experiments have contributed to our current understanding of CR effects on insulin signaling and glucose uptake but future studies are necessary to fully understand the mechanism for CR effects on glucose uptake. Study 1 used 24 month old Fisher Brown Norway (FBN) rats. The 24 month old rat is relatively 60 human years (97) and representative of the onset of old age in humans. Insulin resistance is more prevalent in old age (98) and knowledge with regard to CR effects on skeletal muscle insulin signaling, GLUT4, and glucose uptake in old rats is lacking. The FBN strain has been well characterized for aging and calorie restriction research from the National Institute of Aging (NIA). Results from Study 1 can also be compared to earlier studies that have evaluated the underlying mechanisms for enhanced insulin sensitivity in skeletal muscle from adult FBN rats.

Isolated epitrochlearis and soleus skeletal muscles were used in Study 1 and Study 2 of this thesis. The epitrochlearis and soleus are examples of skeletal muscles of differing fiber types that are widely used in ex vivo incubation experiments. The epitrochlearis muscle assists in extension of the forelimb and originates from the tendon of the m. lattisimus dorsi and inserts on the medial epicondyle of the humerus (99). The

epitrochlearis is 21 to 24 fibers thick (100) which allows for adequate tissue oxygenation to be maintained and is appropriate for ex vivo muscle incubation for glucose uptake and insulin signaling measurements. The predominantly fast-twitch fiber type composition of the rat epitrochlearis (75% Type IIb, 17% Type IIa, and 8% Type I) is representative of the average fiber type composition of the entire rat hindlimb (76% Type IIb, 19% Type IIa, and 5%Type I) (100, 101). The soleus is a postural hindlimb muscle that assists in plantarflexion of the foot. The soleus is composed of primarily slow-twitch Type I fibers (0% Type IIb, 12% Type IIa, and 88% Type I) (81) and is also well characterized for ex vivo incubations (24, 102, 103).

The euglycemic-hyperinsulinemic clamp is used in Study 3 of this thesis. This method is considered the gold standard for assessing insulin sensitivity and allows for in vivo measurements of glucose uptake and insulin signaling. In vivo measurements reflect effects of a physiologic milieu by taking into account the intrinsic muscle properties in addition to extrinsic factors (i.e. skeletal muscle blood flow, neural inputs, and humoral factors). Studies using an in vivo model coupled to an ex vivo model together provides a more complete perspective to that of either model in isolation.

Gaps to be Filled by this Research

The precise cellular mechanism by which CR enhances insulin-mediated cell surface GLUT4 translocation in skeletal muscle has not been fully elucidated. The studies in this dissertation investigate CR changes in expression and phosphorylation of key proteins in the insulin signaling pathway that correspond to an increase in insulin-stimulated glucose uptake in skeletal muscle. **Study 1** assessed the CR effects on insulin

signaling and glucose uptake by isolated skeletal muscles (epitrochlearis and soleus) in old (24 month-old) rats. CR enhances glucose uptake and Akt phosphorylation in isolated epitrochlearis and soleus muscles from adult (9 month-old) rats however AS160 phosphorylation increased only in the epitrochlearis. We hypothesized that CR would enhance glucose uptake and Akt, TBC1D1, and Filamin C phosphorylation in the soleus and epitrochlearis muscles from 24 month-old rats, while AS160 phosphorylation would increase in the epitrochlearis but not in the soleus. **Study 2** investigated whether the CR effect on Akt phosphorylation in isolated soleus muscle from 9 month-old rats is attributable to a rapid and transient activation of proximal insulin signaling (pY-IR, pY-IRS-1, & IRS1-PI3K) in the soleus. We hypothesized that phosphorylation of pY-IR, pY-IRS-1, and PI3K-IRS-1 would be greater for CR versus AL with insulin stimulation at 5 and 15 minutes but not at 50 minutes in the soleus muscle. **Study 3** investigated the effects on insulin signaling and glucose uptake in muscles from 9 month-old rats in vivo, and explored if the CR effects for epitrochlearis and soleus are similar to other fast-twitch (gastrocnemius, plantaris, tibialis anterior) and slow-twitch (adductor longus) muscles. We hypothesized that in vivo stimulation of both predominantly fast-twitch and slow-twitch muscle of CR versus AL would induce greater glucose uptake and Akt phosphorylation while fast twitch muscle of CR versus AL rats would induce greater AS160 phosphorylation and slow twitch muscle from CR versus AL would induce greater TBC1D1 and/or filamin C phosphorylation.

Chapter III

Study 1

Calorie Restriction Enhances Insulin-stimulated Glucose Uptake and Akt Phosphorylation in Both Fast-twitch and Slow-twitch Skeletal Muscle of 24 Month-old Rats

ABSTRACT

Calorie restriction (CR) induces enhanced insulin-stimulated glucose uptake (ISGU) in fast-twitch (type II) muscle from old rats, but the effect of CR on slow-twitch (type I) muscle from old rats is unknown. The purpose of this study was to assess ISGU and phosphorylation of key insulin signaling proteins in isolated epitrochlearis (fast-twitch) and soleus (slow-twitch) muscles from 24 month-old ad libitum (AL) fed and CR (consuming 65% of ad libitum, AL, intake) rats. Muscles were incubated with and without 1.2 nM insulin. CR versus AL rats had greater ISGU, Akt phosphorylation (pAkt) on T308 and S473, and filamin C phosphorylation on S2213 for both muscles incubated with insulin. GLUT4 protein abundance and phosphorylation of the insulin receptor (Y1162/1163), AS160 (T642 and S588) and TBC1D1 (T596) were unaltered by

CR in both muscles. These results implicate enhanced pAkt and pfilamin C as potentially relevant for the mechanism leading to the CR-induced increase in ISGU by the fast-twitch epitrochlearis and slow-twitch soleus of old rats.

INTRODUCTION

Calorie restriction (CR; i.e., consuming ~60-75% of ad libitum, AL, intake) without malnutrition leads to improved function and health in many species (104, 105). One of the hallmarks of CR is an increase in whole body insulin sensitivity (39-41, 106). Given that skeletal muscle accounts for the greatest amount of whole body glucose disposal (2), it is not surprising that CR leads to increased insulin-mediated glucose uptake by skeletal muscle (38, 53, 54) .

Because skeletal muscle is a heterogeneous tissue that is composed of type I (slow-twitch) and type II (fast-twitch) fibers that differ on the basis of contractile and metabolic properties, it is valuable to assess muscles with differing fiber type compositions to gain a full understanding of CR effects. Previous research on adult rats or mice has reported that CR leads to greater insulin-stimulated glucose uptake in skeletal muscles composed primarily of type I (soleus) or type II (epitrochlearis or extensor digitorum longus) fibers (8, 9, 24, 37). In isolated muscle, CR increases insulin-stimulated glucose uptake in the primarily type II epitrochlearis muscle of 23 month-old rats (4, 21), but there have been no reports on insulin-stimulated uptake in a predominantly type I muscle of old rats.

A number of earlier studies have evaluated the underlying mechanisms for improved insulin sensitivity in skeletal muscle from adult rats. Most of the previous

studies have reported little or no CR effect on the abundance of GLUT4 (4, 6, 24, 107), the insulin-regulated glucose transporter protein. We have performed a series of studies that characterized the effects of CR on key insulin signaling proteins in skeletal muscle. Insulin-induced phosphorylation of Akt is essential for insulin-stimulated glucose uptake (68). In this context, it is significant that the most consistent and substantial increase in insulin signaling with CR in skeletal muscle has been an increase in insulin-mediated phosphorylation of Akt (3, 8, 9, 23, 25). Insulin-stimulated Akt phosphorylation was increased by CR in both the soleus (composed of ~90% type I fibers) and the epitrochlearis (~90% type II fibers) in 9 month-old rats (24).

In view of the striking CR effect on Akt phosphorylation in adult animals, it is clearly relevant to identify the Akt substrate(s) that regulate insulin-stimulated glucose uptake. One link between Akt and glucose uptake was discovered by Sano et al. (18, 19) who identified a protein that they called Akt Substrate of 160 kDa (AS160; also known as TBC1D4). They found that AS160 was phosphorylated by Akt in response to insulin, and that the phosphorylation of AS160 modulated insulin-stimulated glucose uptake. They also convincingly demonstrated that Thr642 and Ser588 of AS160 were the most important insulin-regulated Akt phosphosites for insulin-mediated glucose uptake (19). Sharma et al. (24) recently reported a muscle-specific effect of CR in 9 month-old rats: insulin-stimulated Thr642 and Ser588 phosphorylation of AS160 was increased by CR in the epitrochlearis, but not in the soleus. Recently TBC1D1, a paralog of AS160, was shown to be phosphorylated on T596 with insulin stimulation and when Thr596 was substituted to Ala GLUT4 translocation was decreased in 3T3-L1 adipocytes (108). TBC1D1 has also been reported to increase with insulin in rat skeletal muscle (109)

however it is not clear whether TBC1D1 is important for the CR effect on insulin-stimulated glucose uptake in rat skeletal muscle. Another group identified the cytoskeleton protein filamin C (FLNc) as an Akt substrate. FLNc was shown to become phosphorylated on S2213 in response to insulin stimulation, and this insulin-induced phosphorylation was prevented by the presence of the specific PI 3-kinase inhibitor wortmannin in C2C12 myoblasts (76). Filamin C is highly expressed in skeletal muscle and is reported to stabilize actin filament networks on cell membranes (77). Insulin signaling molecules and translocation of GLUT4 transporter vesicles to the cell membrane have been shown to rely on the integrity and maintenance of actin filaments (78, 79). Furthermore, for CR versus AL rats, filamin C phosphorylation in the epitrochlearis muscle stimulated with physiological insulin (Sharma, Arias, Sequea and Cartee, unpublished results).

In contrast to the relatively extensive amount of research on the mechanisms for the CR-induced elevation in insulin-stimulated glucose uptake in muscles from adult animals, little is known about CR effects on insulin signaling in muscle from old rats. A few studies have evaluated the influence of CR on insulin receptor function in skeletal muscle of old rats (6, 26, 27), but these studies did not determine if there were fiber type differences. The effects of CR on Akt, AS160, TBC1D1, or filamin C phosphorylation in insulin-stimulated muscle from old rats, regardless of fiber type, have not been reported. Furthermore, the influence of CR on GLUT4 abundance in the epitrochlearis and soleus of old rats is unknown.

The goal of the current study was to begin to fill some of these gaps in knowledge with regard to CR effects on skeletal muscle insulin signaling, GLUT4 and glucose

transport in old rats. A novel aspect of the experiment was to assess CR effects on insulin-stimulated glucose uptake by both predominantly type I (soleus) and predominantly type II (epitrochlearis) skeletal muscles of old rats. In addition, in the same muscles, we probed the influence of CR on the phosphorylation of key insulin signaling proteins, including the insulin receptor, Akt, AS160, TBC1D1, and filamin C. Finally, we determined if CR resulted in altered expression of GLUT4 protein in the soleus and epitrochlearis of old rats.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, all chemicals were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Hanover Park, IL). Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). Reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Hercules, CA). Anti-phospho-insulin receptor $^{Tyr1162/1163}$ (pIR$^{Tyr1162/1163}$; # 44-504G) and anti-insulin receptor (IR; #AHR0271) were from Invitrogen (Camarillo, CA). Anti-Akt (#9272), anti-phospho Akt^{Ser473} (pAkt^{Ser473}; #9271), anti-phospho Akt^{Thr308} (pAkt^{Thr308}; #9275), anti-GLUT4 (#2213), and anti-rabbit IgG horseradish peroxidase (#7074) were from Cell Signaling Technology (Danvers, MA). Anti-phospho-AS160^{Ser588} (pAS160Ser588; # 3028P2) was from B-Bridge International (Mountain View, CA). Anti-phospho-AS160^{Thr642} (#07-802) and anti-AS160 (#07-741) were from Millipore (Billerica, MA). Anti-phospho-filamin C^{S2213} (#PB-131) was from Kinasource (Scotland, UK). Anti-filamin C (#SC-48496) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-TBC1D1^{T596} was provided

by Dr. Makoto Kanzaki (Tohoku University). 2-Deoxy-D-[^{3}H] glucose ([^{3}H]2-DG) and [^{14}C]mannitol were from Perkin Elmer (Boston, MA).

Animal Care. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. CR and AL male Fischer 344 × Brown Norway, F1 generation rats were obtained at 23 months of age from the National Institute of Aging Calorie Restriction Colony and were individually housed for a month prior to experimentation. CR was initiated at 14 weeks of age with 90% of AL, increased to 75% of AL at 15 weeks, and to 60% of AL at 16 weeks, a level maintained until 23 months of age. Upon arrival at the Michigan animal facility, rats were housed individually in shoebox cages and maintained on a 12:12-h light-dark cycle (lights out at 1700) in specific pathogen-free conditions. The AL group had ad libitum access to the NIH31 chow for the duration of the study. The CR group received NIH31/NIA fortified chow (Test Diet), which contains extra vitamin supplementation to provide CR animals with a level of vitamins similar to that of animals allowed ad libitum access to the NIH31 diet. The CR group received 60-65% of the intake of the AL group daily during the final month of the study. All rats were fed between 1530 and 1630 each day, and food intake of both groups was measured daily. All rats were weighed weekly at the same time of day. Muscle experiments were performed on AL (N=12 and CR (N=13) fed rats at 24 months of age.

Muscle Dissection and Incubation. Food was removed from the cages of all rats on the morning of the experimental day between 07:00 and 08:00 h. Rats were anesthetized

with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) between 10:30 and 13:30 h. Upon loss of pedal reflexes, soleus and epitrochlearis muscles were removed and rapidly rinsed in warm (35^{o}C) Krebs-Henseleit buffer (KHB). Muscles were longitudinally split into strips of similar size for each muscle (2 strips for each epitrochlearis, and 4 strips for each soleus). Muscles strips were subsequently placed in vials containing the appropriate media shaking and continuous gassing (95% O_2/5% CO_2) in a heated (35^{o}C) water bath. In the first incubation step, all muscles were incubated in vials containing 2 ml KHB supplemented with 0.1% bovine serum albumin (BSA), 2mM sodium pyruvate, 6mM mannitol as a rinse step for 30 minutes. In the second incubation step, all muscles were incubated in vials containing 2 ml KHB supplemented with 0.1% BSA, 2 mM sodium pyruvate, 6 mM mannitol, and either 0 nM (basal) or 1.2 nM insulin for 30 min. All muscles were then transferred to a third vial containing 2 ml of KHB/BSA solution, the same insulin concentration as the previous step, 1 mM 2-DG; including a final specific activity of 2.25 mCi/mmol [^{3}H]-2-DG), and 9 mM mannitol (including a final specific activity of 0.022 mCi/mmol [^{14}C]-mannitol) for 20 min. Following the third incubation step, muscles were rapidly blotted on filter paper moistened with ice-cold KHB, trimmed, freeze-clamped using aluminum tongs cooled in liquid nitrogen, and stored at -80^{o}C for later processing and analysis.

Muscle Lysate Preparation. Frozen muscles were weighed, transferred to microfuge tubes and homogenized in ice-cold lysis buffer (1 ml/muscle) using Qiagen a TissueLyser II (Valencia, CA). The lysis buffer contained Tissue Protein Extraction Reagent (Thermo Scientific, Rockford, IL; #78510) supplemented with 1 mM EDTA, 1 mM EGTA, 2.5

mM sodium pyrophosphate, 1 mM sodium vanadate (Na_3VO_4), 1 mM ß-glycerophosphate, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were transferred to microfuge tubes, rotated for 1 h at 4°C, and then centrifuged (15,000 *g*) for 15 min (4°C) to remove insoluble material. Protein concentration was measured using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL; #23225).

Immunoblotting. Equal amounts of protein from each sample were mixed with 6× Laemmli buffer, boiled with SDS loading buffer for 5 min, separated by 10% SDS-PAGE, and then transferred to nitrocellulose. Membranes were rinsed with Tris-buffered saline plus Tween-20 (TBST; 0.14 mol/l NaCl, 0.02 mol/l Tris base, pH 7.6, and 0.1% Tween-20), blocked with 5% bovine serum albumin (BSA) in TBST for 1 h at room temperature and transferred to primary antibody 1:1000 in TBST plus 5% BSA overnight at 4°C. Blots were washed 3 x 5 min with TBST and incubated in buffer containing the appropriate secondary antibody (1:20,000 dilution) for 1 h at room temperature. Membranes were then washed 3 x 5 min with TBST and subjected to enhanced chemiluminescence with West Dura Extended Duration Substrate (Pierce; #34075) for visualization of protein bands. Immunoreactive proteins were quantified by densitometry (AlphaEase FC, Alpha Innotech, San Leandro, CA).

2-Deoxy-D-glucose Uptake. Aliquots (200 µl) of the supernatants were combined in a vial with 10 ml of scintillation cocktail (Research Products International, Mount Prospect, IL) and a scintillation counter (Perkin Elmer, Waltman, MA) was used to

determine ^{3}H and ^{14}C disintegrations per min. These values were used to determine [^{3}H]-2-DG uptake as previously described (110, 111).

Statistical Analysis. A student's t-test was used to compare AL and CR groups. Data are presented as mean ± SEM. A P value < 0.05 was considered statistically significant.

RESULTS

Food Intake, Body Mass and Mass of Muscle Strips

As intended, daily food intake for AL rats (18.4±1.46 g) was greater (P< 0.01) than for CR rats (12.0±1.02 g; 65% of AL), and as expected, body mass was greater (P< 0.05) for AL (533±7 g) versus CR (302.5±1.8 g) rats. Also as expected, the masses of the muscle strips used for ex vivo incubation were greater for the soleus (P< 0.01) of AL (51.7 ±2.5 mg) versus CR (42.0 ±2.4 mg) rats and for the epitrochlearis (P< 0.001) of AL (84.2 ±3.2 mg) versus CR (58.5 ±2.2 mg) rats.

2-Deoxy-D-glucose Uptake

2-DG uptake in the absence of insulin for both the epitrochlearis (P = 0.34) and soleus (P = 0.041) was not significantly different between AL and CR rats (Figure 1). 2-DG uptake with insulin was significantly greater (P < 0.05) for CR versus AL rats in both the epitrochlearis (56% increase) and the soleus (40% increase).

Protein Abundance

Insulin receptor abundance for the CR versus AL group was increased significantly in the epitrochlearis (35% increase; P<0.005) and tended to increase in the

soleus (P=0.056) (Figure 2). Akt abundance was decreased slightly for the CR compared to AL group in the epitrochlearis (9% decrease; P<0.05) (Figure 2). Akt abundance was increased with CR versus AL in the soleus (15% increase; P<0.01). Filamin C abundance did not change with diet in the epitrochlearis. Filamin C was increased (62% increase; P<0.05) with CR versus AL in the soleus muscle. Neither total AS160 nor GLUT4 abundance in either muscle differed for AL versus CR rats (Figure 2).

Insulin Receptor Phosphorylation

In the epitrochlearis, there were no diet effects on insulin receptor phosphorylation either with or without insulin (Figure 3). There were also no significant diet effects on insulin receptor phosphorylation in the soleus with or without insulin.

Akt Phosphorylation

In the absence of insulin, there was no diet effect observed on Akt phosphorylation on either T308 (Figure 4) or S473 (Figure 5) in the epitrochlearis or soleus. In both the epitrochlearis and the soleus under insulin-stimulated conditions, Akt phosphorylation was increased on both T308 (300% in epitrochlearis, P<0.001; and 177% in soleus, P<0.001) and S473 (350% increase in epitrochlearis, P<0.001; and 94% in soleus, P<0.001) for CR versus AL rats.

AS160 Phosphorylation

In both the epitrochlearis and soleus muscle, phosphorylation of AS160 at both the T642 (Figure 6) and S588 (Figure 7) sites did not differ between AL and CR animals in either the absence or the presence of insulin stimulation.

TBC1D1 Phosphorylation

In both the epitrochlearis and soleus muscle, phosphorylation of TBC1D1 at the T596 site did not differ between AL and CR animals in either the absence or the presence of insulin stimulation (Figure 8).

Filamin C Phosphorylation

In the absence of insulin, there was no diet effect observed on FLNc phosphorylation on S2213 in the epitrochlearis or soleus (Figure 9). In both the epitrochlearis and the soleus under insulin-stimulated conditions, FLNc phosphorylation was increased on S2213 (52% in epitrochlearis, $P<0.05$; and 110% in soleus, $P<0.05$) for CR versus AL rats.

DISCUSSION

We previously demonstrated in 23 month-old rats that CR leads to increased insulin-stimulated glucose uptake by the predominantly type II epitrochlearis muscle (4, 21), but the influence of CR on glucose uptake in primarily type I skeletal muscle from old rats had not been previously reported. It was important to fill this gap in knowledge because the insulin-stimulated glucose uptake rates of muscles composed of primarily type I fibers are approximately 2-fold greater than muscles composed of predominantly type II fibers (24, 80, 84). Furthermore, relatively greater age-related insulin resistance has been reported for predominantly type I versus predominantly type II muscles from older rats (84, 112). The most important new findings of the current study for isolated skeletal muscles with a physiological insulin concentration, old (24 month-old) CR versus AL rats were: 1) glucose uptake was enhanced for both type I soleus and type II epitrochlearis; 2) GLUT4 abundance was unchanged in both the soleus and

epitrochlearis; 3) insulin receptor phosphorylation was not altered by diet in either soleus or epitrochlearis; 4) Akt phosphorylation was enhanced in both the soleus and epitrochlearis; 5) AS160 phosphorylation at both T642 and S588 was unchanged by diet in both the soleus and epitrochlearis; 6) TBC1D1 phosphorylation at T596 was unchanged by diet in both soleus and epitrochlearis; and 7) Filamin C phosphorylation at S2213 was greater for CR versus AL rats in both soleus and epitrochlearis.

Total GLUT4 abundance is highly correlated to the capacity for insulin-stimulated glucose uptake in rat skeletal muscle (80). However, total GLUT4 content was unaltered by CR in both the epitrochlearis and the soleus. These results were consistent with the previously published data for the CR effect on GLUT4 in skeletal muscle of old rats. Wang et al reported that CR did not alter total GLUT4 content in the diaphragm of 29 month-old rats (6). Because the diaphragm contracts continuously, and chronic contraction can increase GLUT4 expression (113), the results of the current study for the soleus and epitrochlearis provided valuable new information. The absence of a CR effect on GLUT4 abundance in the soleus of 24 month-old rats is consistent with the results that we previously reported for the soleus of 9 month-old rats (24). GLUT4 abundance in the epitrochlearis was also unaltered by CR in 24 month-old rats, consistent with our previous results for the epitrochlearis of 8 month-old rats (4). However, in a recent study we found a modest (22%) CR-induced increase in GLUT4 abundance in the epitrochlearis of 9 month-old rats (24). CR did not result in altered GLUT4 abundance in the vastus lateralis muscle from adult rhesus monkeys (5). GLUT4 abundance in hindlimb muscles from mice were unaltered (89, 90) or increased (7) in response to CR. Taken together, a number of previous studies have established that even though skeletal

muscle GLUT4 abundance is often unaltered by CR, improved insulin sensitivity is consistently reported with CR in adults. Furthermore, the current results clearly demonstrate that enhanced insulin-stimulated glucose uptake can occur in both type I and type II muscles of old rats in the absence of increased GLUT4 expression. The CR-induced enhancement on insulin-mediated glucose uptake in isolated epitrochlearis muscles from 5.5 month-old rats is attributable to a proportional increase in GLUT4 translocation to the cell surface (3). It seems reasonable to suspect that greater GLUT4 translocation may also mediate the CR-related elevation in insulin-stimulated glucose uptake of muscles from old rats. Because GLUT4 translocation is regulated by the insulin signaling pathway, it was important that we also evaluated CR effects on key insulin signaling steps in muscles from old rats.

There was no CR effect on tyrosine1162/1163 phosphorylation of the insulin receptor for either muscle with a physiologic insulin dose. These tyrosine residues on the insulin receptor's β subunit are major regulatory sites that account for most of the receptor's insulin-mediated autophosphorylation and tyrosine kinase activity (58). Furthermore, replacement of both of these tyrosine residues with phenylalanine residues results in a marked reduction of insulin-stimulated 2DG uptake (58). The lack of a CR-related increase in insulin receptor tyrosine phosphorylation was found despite a moderate (35%) increase for insulin receptor abundance of the epitrochlearis of CR compared to AL rats. These results for insulin receptor abundance are reminiscent of the previously reported modest increase (~19%) in insulin receptor binding capacity (B_{max}) for CR versus AL rats in the diaphragm of 29 month-old rats (6). Zhu et al. (26, 27) reported that after 25 month-old rats were injected with a high dose of insulin into the

portal vein, gastrocnemius muscle (predominantly type II fibers) from CR compared to AL animals had greater insulin receptor tyrosine phosphorylation. In 9 month-old rats, we previously reported no significant CR compared to AL differences for tyrosine phosphorylation of the insulin receptor from soleus or epitrochlearis muscles incubated with a physiologic insulin dose, but there was a CR-related increase in both muscles with a supraphysiologic insulin concentration (24). These results suggest that CR effects on tyrosine phosphorylation of the insulin receptor in skeletal muscle may be insulin dose-dependent. Furthermore, the CR-related amplification of post-receptor signaling and glucose uptake in muscle stimulated by a physiologic insulin dose in the current study do not appear to be attributable to greater tyrosine phosphorylation of the insulin receptor.

We have consistently found a robust CR-induced increase in skeletal muscle Akt phosphorylation in skeletal muscle (both soleus and epitrochlearis) of young adult rats or mice (8-10, 24). The current study was the first to assess the influence of CR on Akt phosphorylation in skeletal muscle from old rats. Consistent with earlier results, CR versus AL rats had a striking increase in insulin-stimulated phosphorylation of the key regulatory sites of Akt (T308 and S473) in both muscles. The small CR effects on Akt abundance (15% increase in soleus and 9% decrease in epitrochlearis) were insufficient to account for the marked (177% increase in soleus and 300% increase in epitrochlearis for T308; 94% increase in soleus and 350% increase in epitrochlearis for S473) increases for Akt phosphorylation with CR. The dramatic increases in Akt phosphorylation with CR at a physiologic insulin dose occurred without an apparent change in upstream signaling at the insulin receptor, and these data are consistent with our previous results for the effect of CR on the soleus and epitrochlearis for 9 month-old rats (24).

AS160 is an Akt substrate and the most distal insulin signaling protein that is clearly implicated in insulin-mediated activation of glucose uptake in skeletal muscle (50, 109). The ability to phosphorylate AS160 on T642 and S588 is essential for the full effect of insulin on GLUT4 translocation and glucose uptake (18, 19, 50, 74). However, we found no evidence for diet-related changes in insulin-stimulated T642 or S588 phosphorylation in either the epitrochlearis or soleus muscle of old rats. Consistent with the current results, we previously reported that T642 phosphorylation of the insulin-stimulated soleus was unaltered by CR for 9 month-old rats (24). The lack of a CR effect on T642 or S588 phosphorylation of AS160 in the epitrochlearis with physiologic insulin in 24 month-old rat differs from the CR-related increase in T642 and S588 for the epitrochlearis of 9 month-old rats (24). The explanation for the different results for T642 and S588 in the epitrochlearis of adult compared to old rats remains to be determined.

TBC1D1 is an Akt substrate and has been implicated in the regulation of glucose uptake(50, 109). In the absence of AS160 phosphoryation, one plausible scenario would be that TBC1D1 may play a role in the CR-mediated increase in insulin-stimulated glucose uptake in old rats. However, we found no evidence of enhanced phosphorylation of T596 in either epitrochlearis or soleus muscles. An alternative possibility is that CR may lead to greater phosphorylation of another Akt substrate that modulates glucose transport. In this context, it was interesting that CR resulted in greater phosphorylation of filamin C in the insulin-stimulated epitrochlearis and soleus muscles. Filamin C was previously identified as a substrate of Akt that in C2C12 myocytes incubated with insulin becomes phosphorylated on Ser2213 (76). Insulin-regulated remodeling of actin filaments has been implicated in the control of both the spatial localization of insulin

signaling proteins and translocation of GLUT4 glucose transporter vesicles (78). Sharma et al. (unpublished results), studying isolated epitrochlearis from 9 month-old rats, also found a CR-induced increase in insulin-stimulated filamin C phosphorylation. They further found that a low dose of a highly selective Akt inhibitor that eliminated the CR-induced increase in Akt phosphorylation also eliminated the CR-induced increases in both glucose uptake and filamin C phosphorylation of the insulin-stimulated epitrochlearis. We demonstrated in this study that insulin-stimulated filamin C phosphorylation at S2213 is enhanced with CR in both the epitrochlearis and soleus muscles from old rats. It should be noted that in the soleus, but not the epitrochlearis, the greater filamin C phosphorylation (110%) in CR rats is likely at least in part attributable to an increase (62%) in total filamin C abundance. If the filamin C phosphorylation of the soleus is expressed as a ratio of total filamin C abundance, there is no longer a statistically significant difference between the AL and CR rats. Regardless, it is unclear if filamin C plays any role in the regulation of glucose transport, and the functional significance of the observed CR-related increase in insulin-mediated phosphorylation of filamin C is unknown.

In conclusion, the most significant new result was that the CR-effect on insulin-stimulated glucose uptake was well-preserved for both type I soleus and type II epitrochlearis of old rats. Furthermore, in both muscles, it seems likely that this increase is secondary, at least in part, to greater insulin-stimulated Akt phosphorylation. However, in neither muscle was insulin-stimulated AS160 (T642 and S588) phosphorylation or TBC1D1 T596 phosphorylation enhanced by CR. Earlier research in adult (9 month-old) rats implicated CR-induced elevation in phosphorylation of Akt and

AS160 as likely important for the greater insuln-stimulated glucose transport in the epitrochlearis muscle (24). The results of the current study suggest that the mechanisms for improved insulin-stimulated glucose uptake in the epitrochlearis with CR may not be completely identical for adult compared to old rats. In both the current study and the previous study of 9 month-old rats, greater insulin-stimulated glucose transport in the soleus was accompanied by greater Akt phosphorylation in the absence of enhanced phosphorylation of either AS160 or TBC1D1. An interesting finding of uncertain functional significance was that CR resulted in greater S2113 phosphorylation of filamin C in insulin-stimulated epitrochlearis and soleus muscles. Future research should focus on determining if the CR effect on filamin C phosphorylation plays a role in greater insulin-stimulated glucose transport in either the epitrochlearis or soleus of old rats.

Acknowledgements

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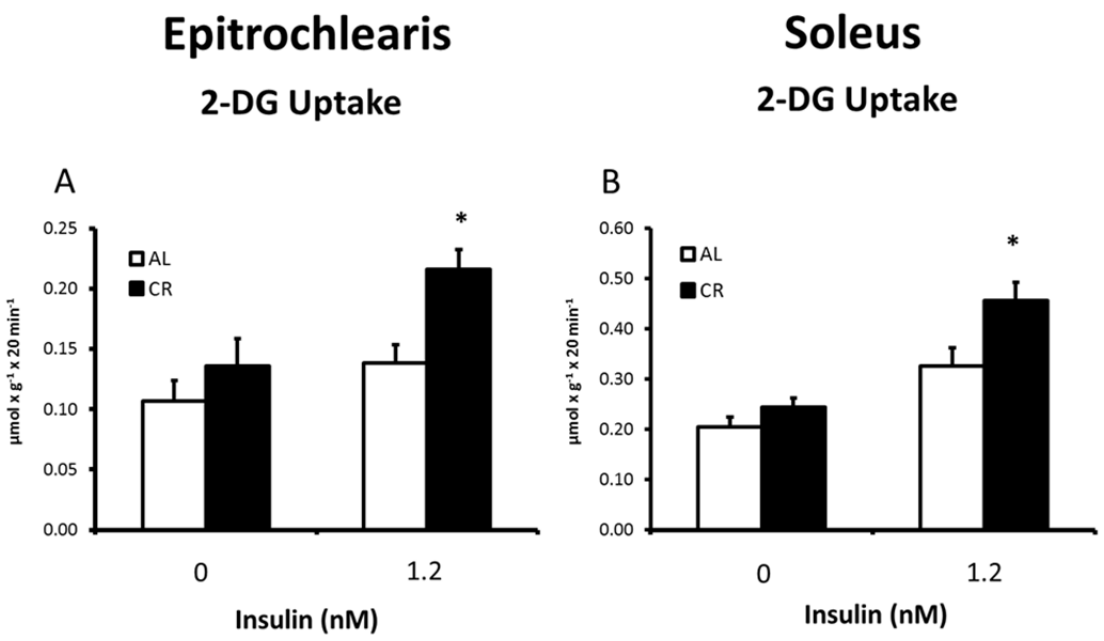

Figure 3.1 2-Deoxy-D-glucose (2-DG) uptake in isolated epitrochlearis and soleus from old rats. 2-DG in epitrochlearis (A) and soleus (B) muscles with 0 or 1.2nM insulin. *$p<0.05$, CR versus AL in the same insulin treatment group. Data are means ± SEM. n=9-11 muscles per diet group and insulin concentration. Open bars represent AL group. Closed bars represent CR group.

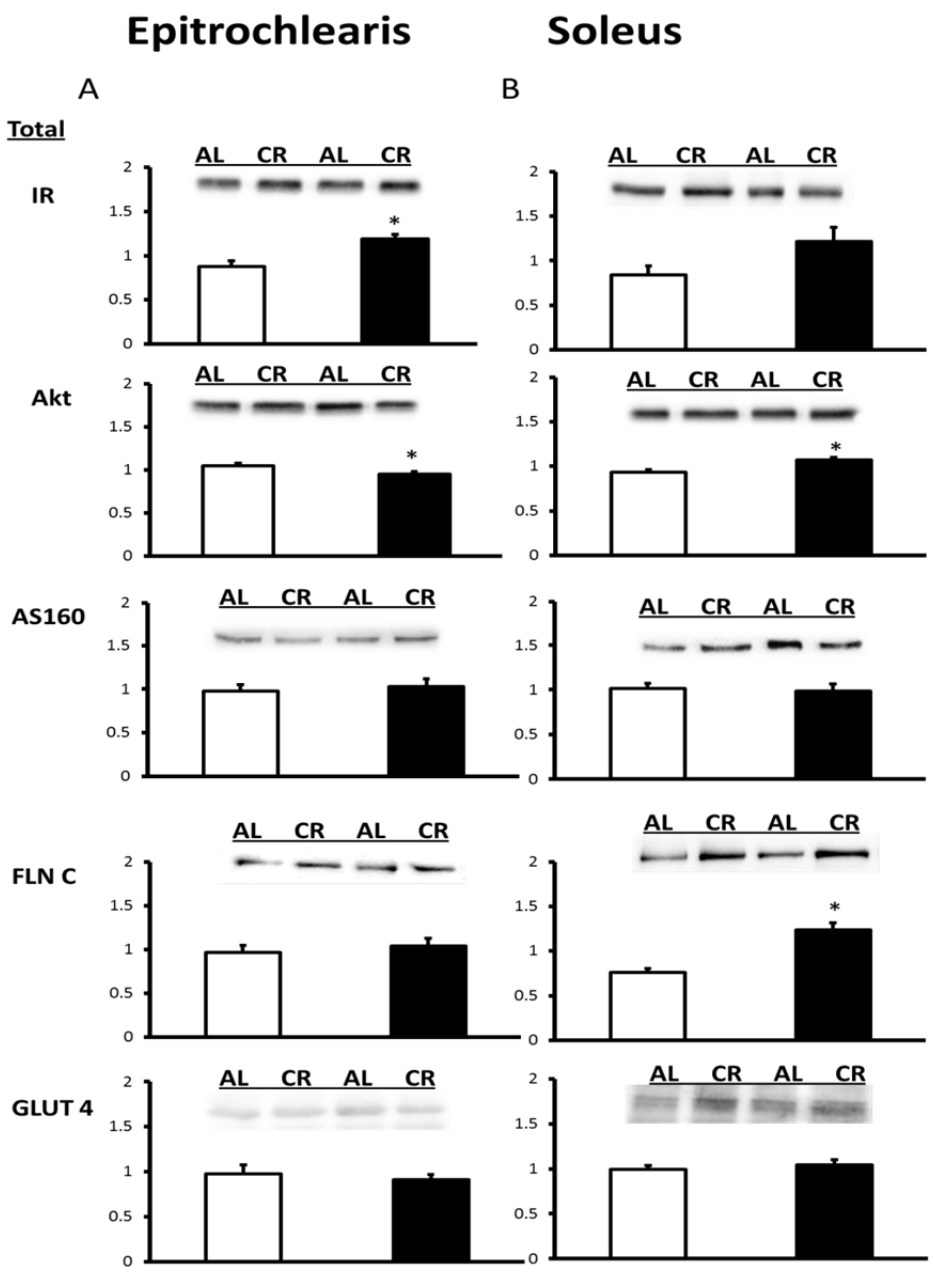

Figure 3.2 Total protein abundance in isolated epitrochlearis and soleus from old rats. Total protein abundance in epitrochlearis (A) and soleus (B) muscles for IR (insulin receptor), Akt, AS160, FLN C (Filamin C) and GLUT4. *$p<0.05$, CR versus AL. Open bars represent AL group. Closed bars represent CR group.

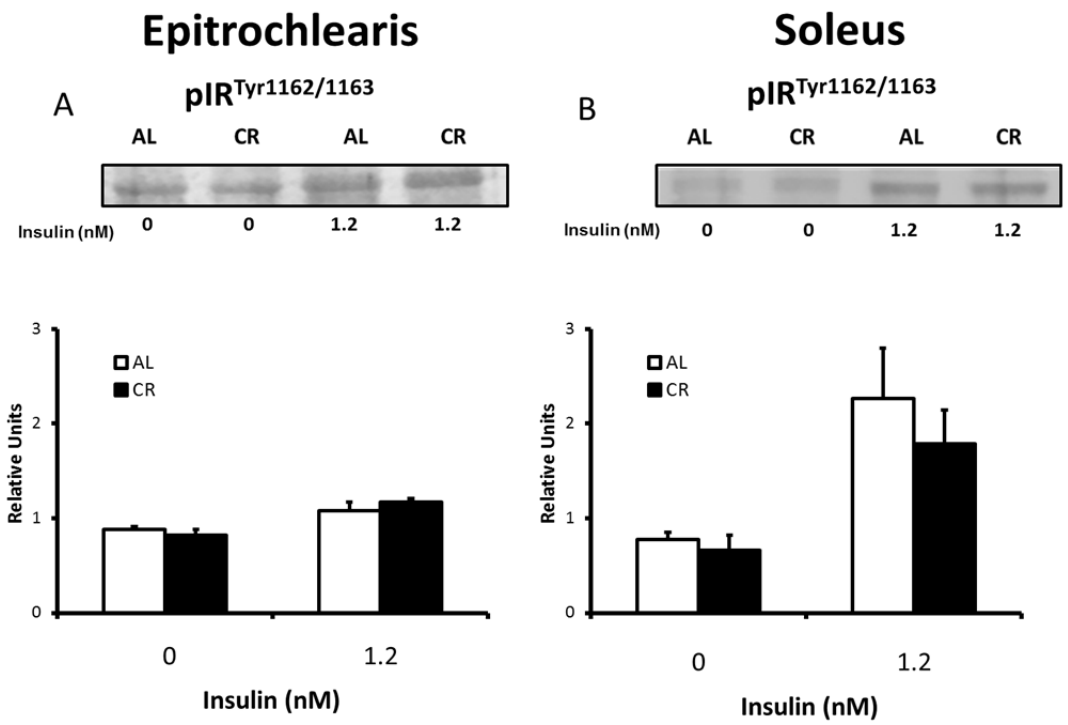

Figure 3.3 Insulin receptor tyrosine phosphorylation ($IR^{Tyr1162/1163}$) in isolated epitrochlearis and soleus from old rats. $IR^{Tyr1162/1163}$ in the epitrochlearis (A) and soleus (B) muscles with 0 or 1.2 nM insulin. *$p<0.05$, CR versus AL in the same insulin treatment group. Data are means ± SEM. n=9-11 muscles per diet group and insulin concentration. Open bars represent AL group. Closed bars represent CR group.

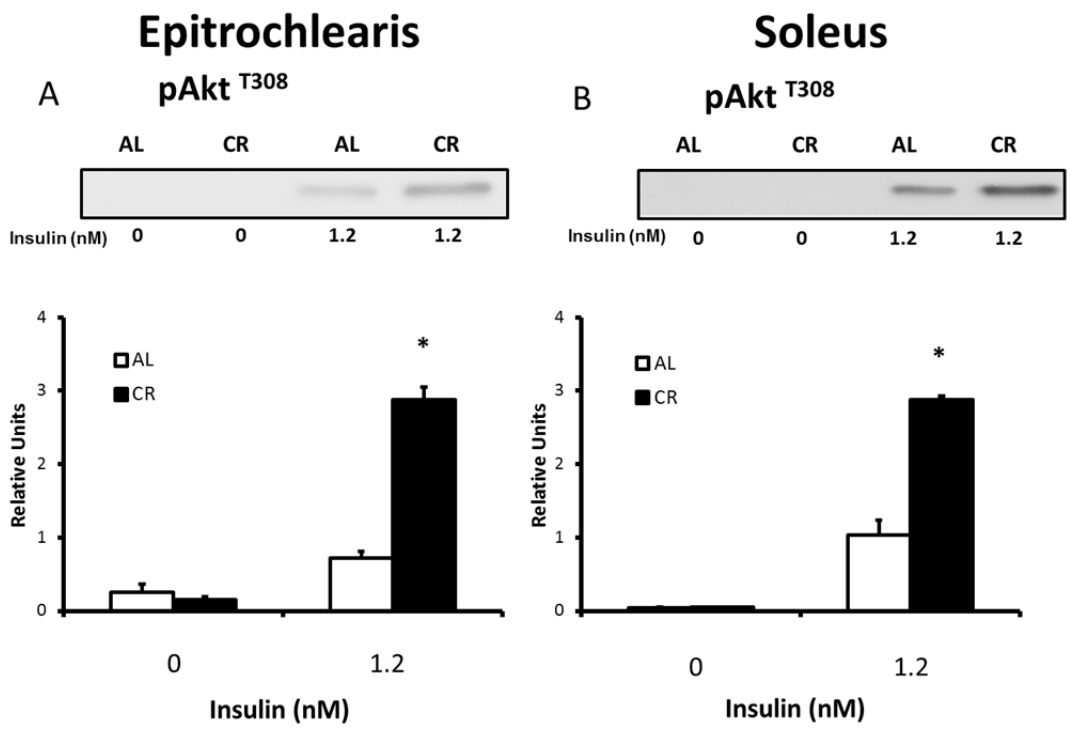

Figure 3.4 Akt^{Thr308} phosphorylation in isolated epitrochlearis and soleus from old rats. Akt^{Thr308} phosphorylation in epitrochlearis (A) and soleus (B) muscles with 0 or 1.2nM insulin . *$p<0.05$, CR versus AL in the same insulin treatment group. Data are means ± SEM. n=9-11 muscles per diet group and insulin concentration. Open bars represent AL group. Closed bars represent CR group.

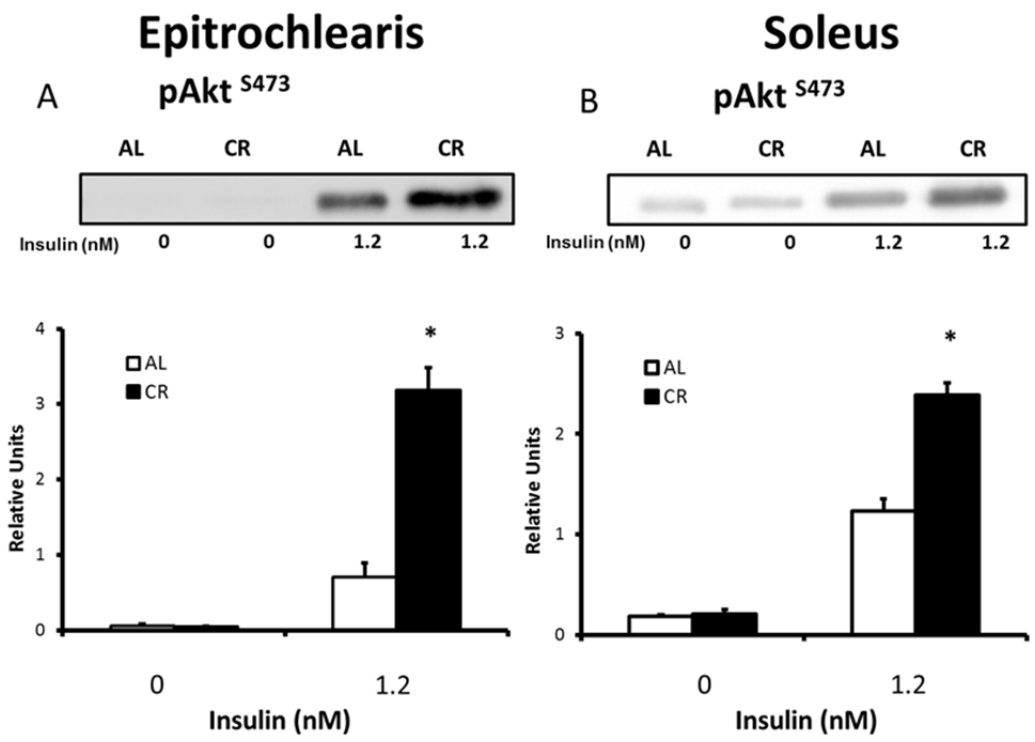

Figure 3.5 Akt^{Ser473} phosphorylation in isolated epitrochlearis and soleus from old rats. Akt^{Ser473} phosphorylation in epitrochlearis (A) and soleus (B) muscles with 0 or 1.2nM insulin. Data are means ± SEM. n=9-11 muscles per diet group and insulin concentration. Open bars represent AL group. Closed bars represent CR group.

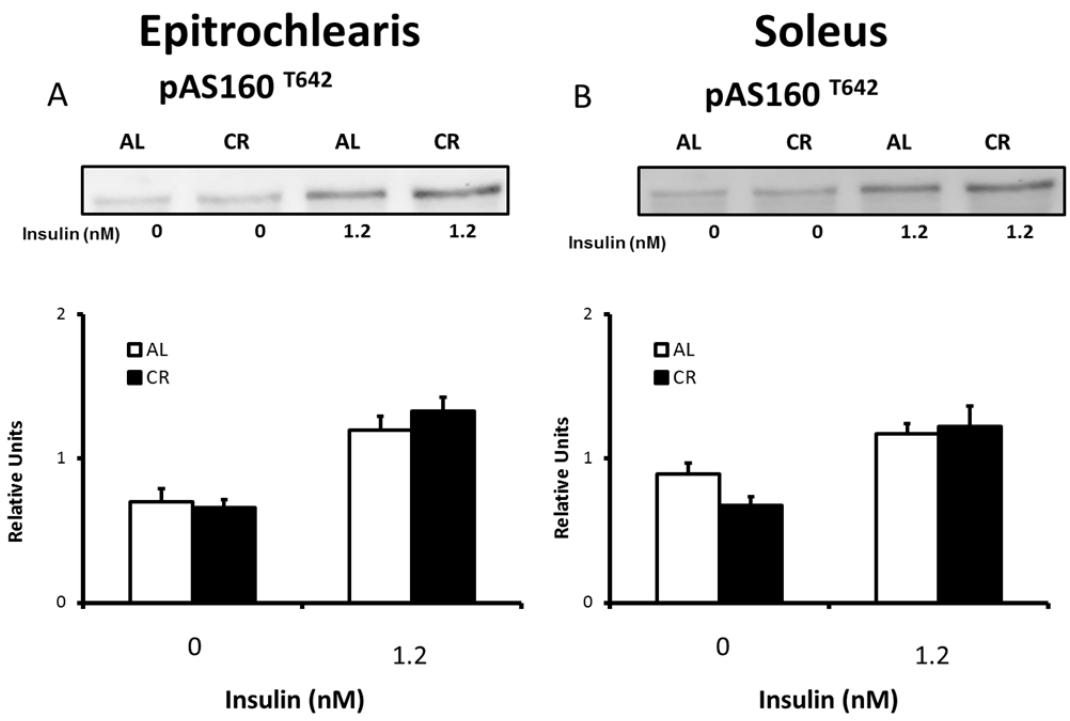

Figure 3.6 AS160^{Thr642} phosphorylation in isolated epitrochlearis and soleus from old rats. AS160^{Thr642} phosphorylation in epitrochlearis (A) and soleus (B) muscles with 0 or 1.2 nM insulin. Data are means ± SEM. n=9-11 muscles per dietary group and insulin concentration. Open bars represent AL group. Closed bars represent CR group.

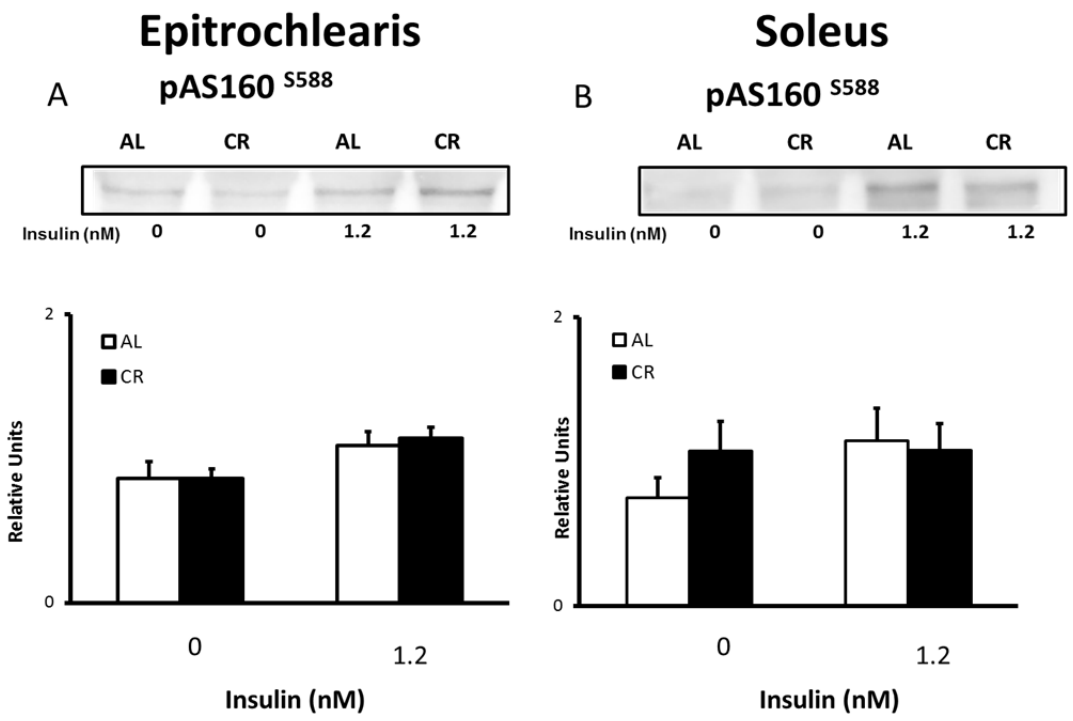

Figure 3.7 AS160^{Ser588} phosphorylation in isolated epitrochlearis and soleus from old rats. AS160^{Ser588} phosphorylation in epitrochlearis (A) and soleus (B) muscles with 0 or 1.2 nM insulin. Data are means ± SEM. n=9-11 muscles per dietary group and insulin concentration. Open bars represent AL group. Closed bars represent CR group.

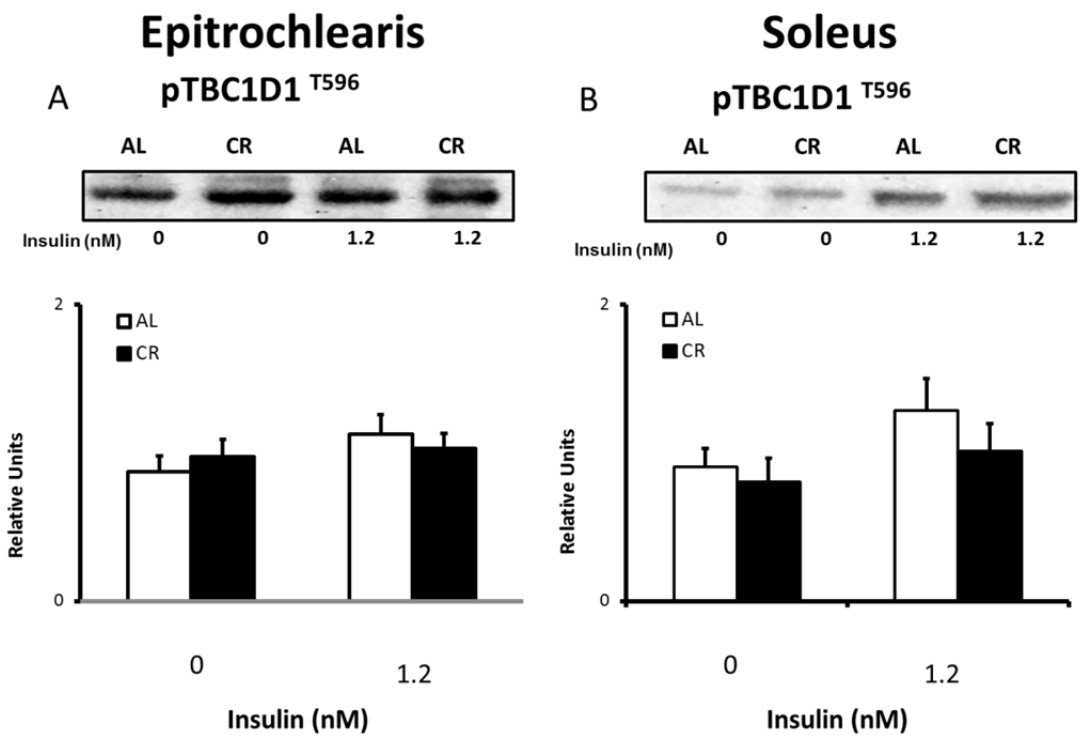

Figure 3.8 TBC1D1^{Thr596} phosphorylation in isolated epitrochlearis and soleus from old rats. TBC1D1^{Thr596} phosphorylation in epitrochlearis (A) and soleus (B) muscles with 0 or 1.2 nM insulin. Data are means ± SEM. n=9-11 muscles per dietary group and insulin concentration. Open bars represent AL group. Closed bars represent CR group.

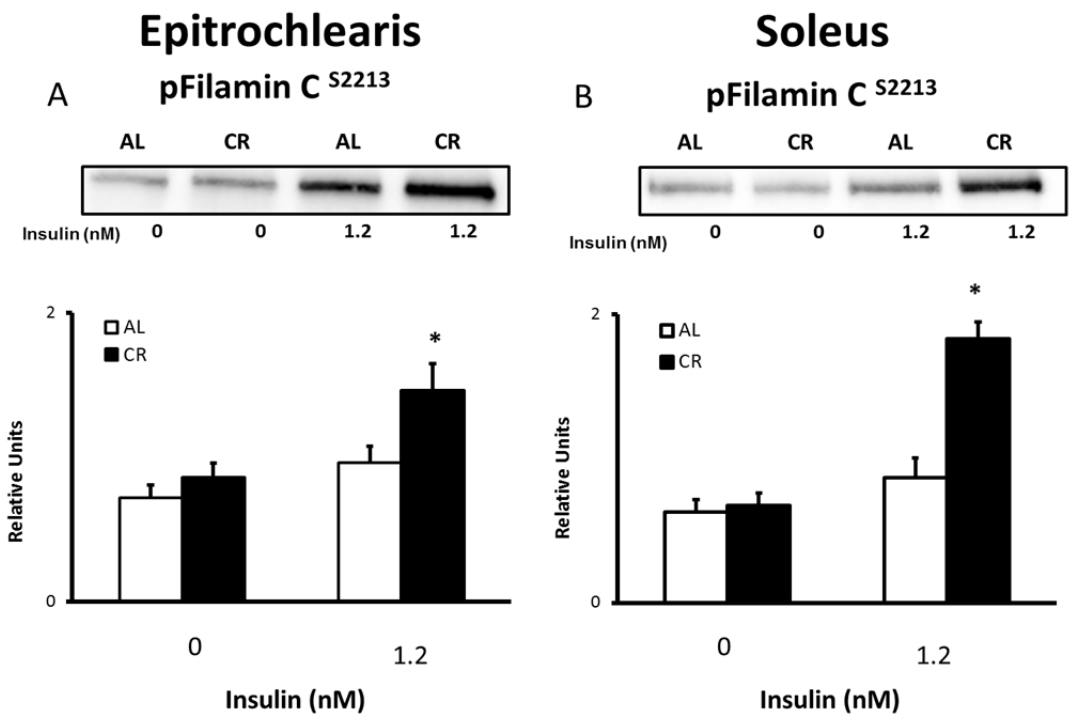

Figure 3.9 Filamin C^{Ser2213} phosphorylation in isolated epitrochlearis and soleus from old rats. Filamin C^{Ser2213} phosphorylation in epitrochlearis (A) and soleus (B) muscles with 0 or 1.2 nM insulin. Data are means ± SEM. n=9-11 muscles per dietary group and insulin concentration. Open bars represent AL group. Closed bars represent CR group.

Chapter IV

Study 2

Comparison of Ad Libitum Fed and Calorie Restricted Rats for the Time Course of Insulin's Activation of Signaling Steps Required for Greater Glucose Transport by Isolated Skeletal Muscle

ABSTRACT

Calorie restriction (CR) induces enhanced insulin-stimulated glucose uptake but the mechanisms responsible are not fully understood. Earlier studies with isolated rat skeletal muscle from rats found CR versus AL animals had greater insulin-stimulated Akt phosphorylation and glucose uptake without diet-effects on key insulin signaling steps proximal to Akt. However, it seemed possible that transient CR effects may have been missed because the signaling effects were made after 45-50 minutes of insulin exposure. Accordingly, the purpose of this study was to evaluate the time course of insulin action on insulin receptor (IR) tyrosine phosphorylation, insulin receptor substrate1 (IRS-1) tyrosine phosphorylation, IRS-1 associated PI3K activity, and Akt phosphorylation in isolated soleus muscles from AL and CR rats with a physiologic dose of insulin for 5, 15, and 50 minutes in soleus muscles from 9 month-old ad libitum (AL) fed and CR

(consuming 65% of ad libitum, AL, intake) Fisher 344/Brown Norway rats. Insulin-stimulated 2-deoxyglucose uptake was significantly increased for CR versus AL rats. There were no significant differences between muscles from CR and AL rats for phosphorylation of the IR on Tyr1162/1163, tyrosine phosphorylation of IRS-1 or IRS-1 associated PI3K at any time point with or without insulin. There was no significant effect of diet for Akt Thr308 and Ser473 phosphorylation at any time point under basal conditions. However, Akt phosphorylation on both Thr308 and Ser473 was significantly increased for the CR versus AL group in muscles that were incubated with insulin for 50 minutes, but not for muscles incubated with insulin for 5 or 15 minutes. The results suggest that the greater Akt phosphorylation and glucose uptake in the muscles from CR rats were attributable to mechanisms independent of diet-related elevations in the levels of IR, IRS-1, and IRS-1 associated PI3K. Our working hypothesis is that the improved Akt activation with CR is related to altered Akt binding to protein partners (TRB3, ClipR-59, CTMP, PHLDB1) that have been reported to regulate Akt phosphorylation.

INTRODUCTION

One of the hallmarks of calorie restriction (CR; i.e., consuming ~60-75% of ad libitum, AL, intake) is an increase in whole body insulin sensitivity (39-41, 106). Given that skeletal muscle accounts for the greatest amount of whole body glucose disposal (2), it is not surprising that CR leads to increased insulin-mediated glucose uptake by skeletal muscle (38, 53, 54). Dean et al. (3) demonstrated that the increased glucose uptake with CR is attributable to a proportional CR-mediated increase in the cell surface GLUT4 at the plasma membrane in insulin-stimulated rat skeletal muscle. The effect of CR on

insulin-stimulated glucose uptake can occur in the absence of increased total GLUT4 protein abundance (88). These results suggest that the mechanism for the CR effect on skeletal muscle glucose transport is related to the specific enhancement of the insulin signaling pathway leading to increase GLUT4 recruitment to the plasma membrane.

Most (24, 28-30, 114), but not all (6) of the published studies that have evaluated CR effects on skeletal muscle insulin receptor (IR) activation with physiologic insulin levels have not found diet-induced differences. In our previous studies, there were no significant diet effects on IR tyrosine phosphorylation (24, 114) or IRS1-PI3K activity (24) with a physiologic insulin concentration. Phosphorylation of Akt (also known as protein kinase B, PKB) on both Ser473 and Thr308 is essential for the full effect of insulin on glucose transport (8). In this context, it is notable that the most consistent result of a series of studies on isolated muscles from CR versus AL rodents has been elevated insulin-induced phosphorylation of Akt for isolated muscles from CR rats (9, 10, 24) and mice (8). These results together suggest elevation in Akt phosphorylation with CR was not attributable to enhancement in insulin receptor or IRS1-PI3K activity. However, enhanced Akt phosphorylation without enhanced IR and IRS-1-PI3Kactivation for CR versus AL was measured at 50 minutes of insulin stimulation so it is possible these observations are attributable to reversal of transient CR effect on proximal steps of insulin signaling that may have reversed by the 50 minute time point**.** The primary aim of this study was to determine the time course of insulin signaling with CR versus AL in response to a physiological insulin dose**.** In isolated soleus muscle from AL and CR rats, we evaluated key upstream insulin signaling proteins [tyrosine phosphorylation of the insulin receptor (IR) and insulin receptor substrate 1(IRS-1) and IRS-1 associated

phosphatidylinositol 3-kinase (PI3K) activity] with insulin stimulation at 5, 15 and 50 minutes. We hypothesized that phosphorylation of $IR^{Tyr1162/1163}$ and $IRS\text{-}1^{Tyr}$, and PI3K-IRS-1 activity would be greater for CR versus AL with insulin stimulation at 5 and 15 minutes, but not at 50 minutes of insulin exposure. We also measured insulin-stimulated glucose uptake at 50 minutes of insulin exposure to confirm the expected CR effect on insulin sensitivity.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, all chemicals were purchased from Sigma Chemical (St. Louis, MO) or Fisher Scientific (Hanover Park, IL). Human recombinant insulin was obtained from Eli Lilly (Indianapolis, IN). Tissue Protein Extraction Reagent (#78510) was from Thermo Scientific (Rockford, IL). $MILLIPLEX_{MAP}$ cell signaling buffer and detection Kit (#48-602), MILLIPLEX phospho-MAPmates for $IR^{Tyr1162/1163}$(#46-688), $IRS1^{Tyr}$ (#46-627), Akt^{Ser473} (#46-601), Akt^{Thr308} (#46-645), total MAPmates for Akt (#46-605), IR (#46-687), and IRS1 (#46-628) were all purchased from Millipore (Billerica, MA). Reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Hercules, CA). Anti-Akt (#9272), anti-phospho Akt^{Ser473} ($pAkt^{Ser473}$; #9271), and anti-rabbit IgG horseradish peroxidase (#7074) were from Cell Signaling Technology (Danvers, MA). Pierce BCA (bicinchoninic acid) protein assay (#23225) and West Dura Extended Duration Substrate (#34075) were from Pierce Biotechnology (Rockford, IL). Scintillation counter, 2-Deoxy-D-[^{3}H] glucose ([^{3}H] 2-DG), [^{14}C]mannitol, and γ-[^{32}P]ATP were from Perkin Elmer (Boston, MA). Scintillation cocktail # 111195 was from Research Products International (Mount Prospect, IL). A-

Sepharose beads (#17-0469-01) were from GE Healthcare (Piscataway, NJ). Phosphatidylinositol (PI) was from Avanti Polar Lipids (Alabaster, AL). TLC plates #4865-821 were from Whatman (Piscataway, NJ).

Animal Care. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. CR and AL male Fischer 344 × Brown Norway, F1 generation rats were obtained at 8 months of age from the National Institute of Aging(NIA) Calorie Restriction Colony and were individually housed for a month prior to experimentation. CR was initiated with NIA colony at 14 weeks of age with 90% of AL, increased to 75% of AL at 15 weeks, and to 60% of AL at 16 weeks, a level maintained until 8 months of age. Upon arrival at the Michigan animal facility at 8 months-old, rats were housed individually in shoebox cages and maintained on a 12:12-h light-dark cycle (lights out at 1700) in specific pathogen-free conditions. The AL group had ad libitum access to the NIH31 chow for the duration of the study. The CR group received NIH31/NIA fortified chow (Test Diet), which contains extra vitamin supplementation to provide CR animals with a level of vitamins similar to that of animals allowed ad libitum access to the NIH31 diet. The CR group received 60-65% of the intake of the AL group daily during the final month of the study. All rats were fed between 15:30 h and 16:30 h each day, and food intake of both groups was measured daily. Muscle experiments were performed on AL (N=14 and CR (N=14) rats at 9 months of age.

Muscle Dissection and Incubation. Food was removed from the cages of all rats on the morning of the experimental day between 07:00 and 08:00 h. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) between 10:30 and 13:30 h. Upon loss of pedal reflexes, soleus and epitrochlearis muscles were removed and rapidly rinsed in warm (35^{o}C) Krebs-Henseleit buffer (KHB). Muscles were longitudinally split into strips of similar size for each muscle (4 strips for each soleus). Muscles strips were subsequently placed in vials containing the appropriate media shaking and continuous gassing (95% O_2/5% CO_2) in a heated (35^{o}C) water bath. In the first incubation step, all muscles were incubated in vials containing 2 ml KHB supplemented with 0.1% bovine serum albumin (BSA), 2mM sodium pyruvate, 6mM mannitol as a rinse step for 30 minutes. In the second incubation step, muscles were incubated in vials containing 2 ml KHB supplemented with 0.1% BSA, 2 mM sodium pyruvate, 6 mM mannitol, and either 0 nM (basal) or 1.2 nM insulin for 5 min, 15 min, or 30 min. The muscles incubated in the second step for 5 minutes and 15 minutes were then rapidly blotted on filter paper moistened with ice-cold KHB, trimmed, freeze-clamped using aluminum tongs cooled in liquid nitrogen, and stored at -80^{o}C for later processing and analysis. The remaining muscles were either transferred to a third vial containing 2 ml of KHB/BSA solution, the same insulin concentration as the previous step with 0.1% BSA, 2 mM sodium pyruvate, 6 mM mannitol for 20 minutes or to assay for 2-Deoxy-D-glucose uptake transferred to a third vial containing 2 ml of KHB/BSA solution, the same insulin concentration as the previous step, 1 mM 2-DG; including a final specific activity of 2.25 mCi/mmol [^{3}H]-2-DG), and 9 mM mannitol (including a final specific activity of 0.022 mCi/mmol [^{14}C]-mannitol) for 20 min. Following the

third incubation step, the muscles were rapidly blotted on filter paper moistened with ice-cold KHB, trimmed, freeze-clamped using aluminum tongs cooled in liquid nitrogen, and stored at -80^{o}C for later processing and analysis.

Muscle Lysate Preparation. Frozen muscles were weighed, transferred to microfuge tubes and homogenized in ice-cold lysis buffer (1 ml/muscle) using Qiagen a TissueLyser II (Valencia, CA). The lysis buffer contained Tissue Protein Extraction Reagent supplemented with 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate (Na_3VO_4), 1 mM ß-glycerophosphate, 1 µg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Homogenates were transferred to microfuge tubes, rotated for 1 h at 4°C, and then centrifuged (15,000 *g*) for 15 min (4^{o}C) to remove insoluble material. Protein concentration was measured using the bicinchoninic acid method.

*Immunoblotting***.** Equal amounts of protein from each sample were mixed with 6× Laemmli buffer, boiled with SDS loading buffer for 5 min, separated by 10% SDS-PAGE, and then transferred to nitrocellulose. Membranes were rinsed with Tris-buffered saline plus Tween-20 (TBST; 0.14 mol/l NaCl, 0.02 mol/l Tris base, pH 7.6, and 0.1% Tween-20), blocked with 5% bovine serum albumin (BSA) in TBST for 1 h at room temperature and transferred to primary antibody 1:1000 in TBST plus 5% BSA overnight at 4°C. Blots were washed 3 x 5 min with TBST and incubated in buffer containing the appropriate secondary antibody (1:20,000 dilution) for 1 h at room temperature. Membranes were then washed 3 x 5 min with TBST and subjected to enhanced

chemiluminescence with West Dura Extended Duration Substrate for visualization of protein bands. Immunoreactive proteins were quantified by densitometry (AlphaEase FC, Alpha Innotech, San Leandro, CA).

2-Deoxy-D-glucose Uptake. Aliquots (200 μl) of the supernatants were combined in a vial with 10 ml of scintillation cocktail (Research Products International, Mount Prospect, IL) and a scintillation counter was used to determine ^{3}H and ^{14}C disintegrations per min. These values were used to determine [^{3}H]-2-DG uptake as previously described (110, 111).

IRS-1-associated PI3K activity. IRS1-PI3K activity in the soleus muscle was determined as previously described (23, 24). After addition of 3 μg of anti-IRS-1 antibody to 500 μg of supernatant protein from each muscle sample, the immunocomplexes were allowed to form overnight at 4°C with slow rotation. Then 100 μl of protein A-Sepharose beads were then added to each aliquot, and samples were rotated for 2 h at 4°C. Samples were centrifuged at 2,000 *g* to pellet the protein A-Sepharose immunocomplex. Each immunopellet was washed three times with *Buffer 1* (PBS, pH 7.5, containing 1% NP-40 and 100 μM Na_3VO_4), three times with *Buffer 2* (100 mM Tris, pH 7.5, 500 mM $LiCl_2$, and 100 μM Na_3VO_4), and twice with *Buffer 3* (10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA, and 100 μM Na_3VO_4). After the immunopellet was washed, the entire buffer was removed, and the immunopellet was resuspended in 40 μl of the Tris·NaCl, pH 7.5, buffer containing 20 μg of phosphatidylinositol and 100 mM $MgCl_2$. The reaction was initiated at room temperature

by addition of 5 µl of a phosphorylation mixture containing 880 µM ATP and 30 µCi of γ-[^{32}P]ATP. After 40 min with continuous rotation at 30°C, the reaction was stopped by sequential addition of 20 µl of 8 N HCl and 160 µl of chloroform-methanol (1:1). The reaction mixture was vortexed for 5 min and then centrifuged at 15,000 *g* for 2 min; 50 µl of the organic phase containing the reaction products was spotted onto a TLC plate. The products were resolved in a chloroform-methanol-water-ammonium hydroxide (60:47:11.3:2) solution and visualized by autoradiography. The spots corresponding to the PI phosphorylated product were scraped from the TLC plate and counted in a scintillation counter.

Luminex. Luminex analysis was performed by the Luminex L200 instrument (Luminex, Austin, TX), as described previously (115). An aliquot of lysate from each tissue was used to determine the phosphorylation status of insulin signaling proteins (IR$^{Tyr1162/1163}$, IRS-1^{Tyr}, Akt^{Ser473}, Akt^{Thr308}) using a commercially available kit (#48-602, Millipore). Another aliquot from each tissue sample was used in a second multiplex assay to determine the relative abundance of proteins (IR, IRS1, Akt).

Statistical Analysis. 3-way ANOVA was used to determine the main effects of Insulin (0 or 1.2nM), Time (5, 15 or 15 min) and Diet (AL or CR) on insulin signaling in the soleus muscles (pIR $^{Tyr1162/1163}$, pIRS-1^{Tyr}, PI3K-IRS-1 activity, pAkt^{Ser473}, and pAkt^{Thr308}). A student's t-test was used to compare AL and CR groups for 2-DG uptake and insulin signaling matched for both time-point and insulin dose. Data are presented as mean ± SEM. A P value ≤ 0.05 for was considered statistically significant.

RESULTS

2-Deoxy-D-Glucose Uptake

2-DG uptake in the absence of insulin was not significantly different between AL and CR rats. 2-DG uptake with insulin was significantly greater ($P<0.05$) for CR versus AL rats (Figure 1).

$IR^{Tyr1162/1163}$ Phosphorylation Time Course

ANOVA revealed that there was a non-significant trend for a main effect of insulin ($P=0.058$), but no evidence for main effects of either time or diet on IR Tyr1162/1163 phosphorylation (Figure 2). Based on t-tests comparing AL versus CR values at each insulin dose and time, there were no significant effects of diet on IR phosphorylation on Tyr1162/1163, either in the absence or presence of insulin (Table1).

$IRS\text{-}1^{Tyr}$ Phosphorylation Time Course

ANOVA revealed that there was significant main effect insulin ($P<0.005$), but no evidence for main effects of either time or diet on IRS-1 Tyr phosphorylation (Figure 2). Based on t-tests comparing AL versus CR values at each insulin dose and time, there were no significant diet effects on IRS-1 phosphorylation, either in the absence or presence of insulin (Table 1).

IRS-1 associated PI3K Activity Time Course

ANOVA revealed that there was a significant main effect of insulin ($P<0.001$), but no evidence for main effects of either time or diet on IRS-1 associated PI3K activity (Figure 2). Based on t-tests comparing AL versus CR values at each insulin dose and

time, there was no significant diet effect seen on IRS-1 associated PI3K activity, either in the absence or presence of insulin (Table 1).

Akt^{Thr308} Phosphorylation Time Course

ANOVA revealed that there were significant main effects of insulin ($P<0.001$), time ($P<0.05$), and diet ($P<0.05$) on Akt Thr308 phosphorylation (Figure 3). Based on t-tests comparing AL versus CR values in the absence of insulin at each time, there were no significant diet effects on Akt Thr308 phosphorylation (Table 1). The results of the t-tests comparing AL versus CR values at each time in the presence of insulin also revealed no significant diet effects on Akt phosphorylation on Thr308 at 5 or 15 minutes, but at 50 minutes, Akt phosphorylation on Thr308 was significantly greater ($P<0.05$) for CR versus AL rats (Table 1).

Akt^{Ser473} Phosphorylation Time Course

ANOVA revealed that there were significant main effects of insulin ($P<0.001$) and diet ($P<0.05$), but not time on Akt Ser473 phosphorylation (Figure 3). Based on t-tests comparing AL versus CR values in the absence of insulin at each time, there were no diet effects for Akt Ser473 phosphorylation (Table 1). The results of the t-tests comparing AL versus CR values at each time in the presence of insulin also revealed no significant diet effects on Akt phosphorylation on Ser473 at 5 or 15 minutes, but at 50 minutes, Akt phosphorylation on Thr308 was significantly greater ($P<0.05$) for CR versus AL rats (Table 1).

DISCUSSION

Prior research has shown that CR can enhance insulin-stimulated glucose uptake in isolated muscle, and this effect consistently occurs concomitant with an increase in phosphorylation of Akt. Several earlier studies of CR effects that used isolated rat skeletal muscle reported that CR did not induce significantly greater activation of key upstream insulin signaling steps (tyrosine phosphorylation of IR or IRS1, and IRS-1-associated PI3K activity) that regulate Akt phosphorylation (3, 24, 25). These results suggest that the mechanism for the CR-induced increase in Akt activation is not dependent on greater activation of these important proximal insulin signaling steps. However, because earlier studies had only evaluated the soleus at a single time-point of insulin exposure (50 minutes), it seemed possible that a transient CR-related activation might have been missed. Therefore, we evaluated the time course of insulin action on insulin receptor (IR) tyrosine phosphorylation, insulin receptor substrate1 (IRS-1) tyrosine phosphorylation, IRS-1 associated PI3K, and Akt in isolated soleus muscles from AL and CR rats with a physiologic dose of insulin for 5, 15, and 50 minutes. As expected, skeletal muscle 2-DG uptake was significantly increased with insulin for CR versus AL. There was no significant increase between muscles from CR and AL rats for phosphorylation of the IR on Tyr1162/1163 at any time point with or without insulin. Tyrosine phosphorylation of IRS-1 was also not significantly different between CR and AL rats regardless of insulin dose or time point. There was also not significantly greater IRS-1 associated PI3K for CR versus AL at any time point with or without insulin. There was no significant effect of diet for Akt Thr308 and Ser473 phosphorylation at any time point under basal conditions. However, phosphorylation of Akt on both Thr308 and Ser473 was significantly increased for the CR versus AL group in muscles that were

incubated with insulin for 50 minutes, but not for muscles incubated with insulin for 5 or 15 minutes.

Muscle 2-DG uptake was increased with 50 minutes of insulin exposure for CR versus AL rats, indicating that, as expected, there was enhanced insulin sensitivity in the soleus muscles of the CR group. The CR effect on insulin-stimulated glucose uptake has been seen numerous times before in the soleus and epitrochlearis muscles of rats (9, 10, 24) and in the soleus, epitrochlearis and extensor digitoroum longus muscles of mice (8, 37). The increased insulin-mediated glucose uptake is attributable to an increase in cell-surface GLUT4 (3) and can occur without an increase in overall GLUT4 abundance (4, 84, 114).

IR phosphorylation has previously been shown to be similar between AL and CR rats with 50 minutes of insulin stimulation in 9 month and 24 month-old rats of the same strain used in this study (24, 114). It was not clear, however, if transiently greater insulin receptor activation with CR may have occurred at an earlier timepoint and reversed by 50 minutes. In this study we show that IR phosphorylation at Tyr1162/1163 is similar between AL and CR rats at 5, 15, and 50 minutes of insulin stimulation. These tyrosine residues on the insulin receptor's β subunit are major regulatory sites that account for most of the receptor's insulin-mediated autophosphorylation and tyrosine kinase activity (58). Based on the current results, it appears that CR's effects on more distal signaling in isolated soelus muscles from rats are attributable to some mechanism other than greater IR phosphorylation at Tyr1162/1163.

The effect of CR on IRS-1 tyrosine phosphorylation has apparently not been studied in isolated rodent skeletal muscle with a submaximal insulin dose, but Dean and

Cartee (23) studied AL and CR rats that received a portal injection of a supraphysiologic insulin dose. Gastrocnemius muscle pan-tyrosine phosphorylation of IRS-1 was greater for insulin-injected CR versus AL rats. Wang et al. (116) evaluated pIRS-1^{Tyr612} in calorie restricted and ad libitum cytomolgus monkeys at 5, 20, and 40 minutes of insulin infusion (plasma insulin of ~1.5 nM) during a euglycemic-hyperinsulinemic clamp. At 40 minutes of insulin infusion, there was no significant CR versus AL effect on vastus lateralis muscle pIRS-1^{Tyr612}. However at both 5 and 20 minutes there were significant increases in IRS-1 phosphorylation at Tyr612. In our study we found no evidence for a CR versus AL increase in IRS-1 tyrosine phosphorylation at either 5, 15, or 50 minutes in the rat soleus muscle. It is important to note that the current study evaluated pan-tyrosine phosphorylation whereas Wang et al. looked at the specific Tyr 612 site. Pan-tyrosine IRS-1 phosphorylation was used to capture the multiple tyrosine phosphorylation sites on IRS-1 that are activated by insulin. It remains to be determined if pIRS-1^{Tyr612} increases with CR versus AL in isolated rat soleus muscles. It is also possible that other differences between the current study the study of Wang et al. account for the differing results on the activation of tyrosine phosphorylation in skeletal muscle (e.g., differences in the species studied, specific muscles studied, in vivo versus ex vivo, etc.)

Previous studies evaluating the effect of CR on IRS-1-, IRS-2- or phosphotyrosine-associated PI3K activity have reported no significant differences between CR versus AL rats in muscle exposed to 45 to 50 minutes with submaximally effective insulin doses in isolated epitrochlearis muscles from rats (24, 25) suggesting that the enhanced insulin sensitivity with CR is dependent on effects occurring downstream of IRS-1-PI3K signaling. Dean et al. (3) evaluated IRS-1-PI3K activity in

with 4, 8, and 20 minutes of a supraphysiological insulin dose (120nM) and did not find a significant difference with CR versus AL at any time point. However, a recent study by Schenk et al. (117) in isolated soleus muscle from mice found that phosphotyrosine-associated PI3K activity increased with CR versus AL using 60 μU/ml insulin with10 minutes of insulin stimulation. In CR versus AL, there was no change on insulin stimulation of IRS1-, IRS2-, or phosphotyrosine-PI3K in rat epitrochlearis (25). Wang et al. (116) reported that in vastus lateralis muscle from calorie restricted monkeys, IRS-1 associated PI3K activity was greater for CR versus AL with 5, 20, and 40 minutes of insulin stimulation. The current study and earlier studies with rats have not found significant CR effects on PI3K activity, whereas two studies that found CR effects on PI3K activity studied other species (mice and monkeys). It remains possible that there may be species differences in PI3K activation with insulin for CR versus AL animals.

In the current study, no significant differences between AL and CR rats were detected for IRS-1-PI3K activity in isolated rat soleus muscle at 5, 15, and 50 minutes with 1.2 nM insulin. At least in isolated rat skeletal muscles, the results of multiple studies have not identified significant CR-induced elevations in IRS-1 PI3K activity, and the current study has extended earlier research by providing evidence at earlier time-points than previously evaluated with a physiologic insulin dose. The lack of a statistically detectable CR effect on proximal signaling concomitant with a consistently significant CR effect on Akt could potentially be related to differences in the technical precision of the methods used to quantify the different insulin signaling steps. The dynamic range was smaller and the variability was greater for proximal signaling markers versus Akt signaling. It is possible that the lack of a statistically detectable CR effect of

proximal signaling steps in this study was due to insufficient sample size and statistical power. Only 3 time points were assessed and a difference may have been missed at an intermediate time point that was not studied. However, it is notable that the results of the current study are consistent with earlier studies for rat skeletal muscle. Previously, no CR effect on IRS1-, IRS2, or phosphotyrosine PI3K was reported with 3 nM insulin in the epitrochlearis (25). Sharma et al. (24) observed no CR effect on PI3K activity together with no evidence for a CR effect on atypical PKC activity. The lack of any change in atypical PKC activity with CR, a step that is dependent on the proximal signaling events that also regulate Akt (including insulin receptor and IRS-1 tyrosine phosphorylation and IRS-1-PI3K activity) is consistent with a lack of a general increase in proximal signaling with CR, and it supports the idea of CR does not uniformly enhance all signaling events that are modulated by insulin via activation of PI3K activity.

The most consistent CR effect on insulin signaling in skeletal muscle that has been observed in numerous studies is greater Akt phosphorylation on the two key sites that regulate Akt activity (Thr308 and Ser473) (8-10, 24). This effect has been repeatedly seen with both supraphysiologic and physiological levels of insulin. Cho et al. (68) showed that Akt2 is the critical isoform for regulating most of the insulin-stimulated glucose transport in skeletal muscle. In the current study, consistent with earlier research, Akt phosphorylation on Thr308 and Ser473 was significantly increased for CR versus AL at 50 minutes of insulin-treatment. Akt phosphorylation was not significantly different between the diet groups at either 5 or 15 minutes, although there was a trend for higher values on Ser473 at both times and Thr308 at 15 minutes. In insulin-infused cynomolgus monkeys, there were significant CR-related increases in Akt Ser473 phosphorylation at 5,

20 and 40 minutes (116). An earlier time of onset for the CR effect on pAkt with in vivo insulin infusion would be favored by the delivery of insulin via the vascular system as opposed to the diffusion of insulin from media to the tissue in isolated muscles, but there may also be other in vivo factors that would favor the more rapid time course for the CR-effect.

The lack of evidence for significant CR effects on proximal insulin signaling steps in isolated rat skeletal muscle at various early time-points begs the question, what might account for the greater Akt that is consistently found in rat skeletal muscle? One possibility is that CR may influence the subcellular localization of Akt. Full activation requires Akt insertion into the plasma membrane. CR may increase the number of Akt molecules at the plasma membrane leading to greater activation. However, it is unclear what might account for such a change in Akt localization with CR. There is evidence that CR may alter the binding of Akt to proteins that lead to greater phosphorylation thus enhancing Akt signaling. In the epitrochlearis muscles from 9 mo-old rats, CR versus AL animals had greater HSP90-bound to Akt (based on co-immunoprecipitation) concomitant with increased Akt phosphorylation. However, there was no evidence of increased HSP90-Akt association in the soleus of CR rats in this earlier study. It remains possible that CR may be improving Akt signaling in the soleus through alterations in the binding to other protein partners. Because phosphorylation of proteins depends on the balance between the actions of kinases and phosphatases, another possibility is that CR reduces the rate of Akt dephosphorylation. However, Sharma et al. (24) found in soleus and epitrochlearis muscles from 9 mo-old rats that there was no CR-related difference in

Akt association with Protein Phosphatase 2A (PP2A), the key Ser/Thr protein phosphatase that dephosphorylates Akt.

In summary, the current study confirms earlier research that demonstrated 50 minutes of exposure to a physiologic insulin dose is sufficient for enhanced glucose uptake concomitant with increased Akt phosphorylation in isolated muscles from CR versus AL rats. We predicted that upstream signaling would be transiently (at 5 and/or 15 minutes) greater for CR versus AL rats. However, muscles from CR versus AL rats did not differ significantly for $IR^{Tyr1162/1163}$ phosphorylation, IRS-1^{Tyr} phosphorylation, or IRS-1-associated PI3K activity, at any of the times tested (5, 15 and 50 minutes) suggesting that the greater Akt phosphorylation and glucose uptake in the muscles from CR rats were attributable to another mechanism. Our working hypothesis is that the improved Akt activation with CR is related to altered Akt binding to protein partners that regulate Akt phosphorylation. It will be important for future research to test this idea by evaluating Akt binding to proteins that have been reported to favor greater Akt phosphorylation [e.g.PHLDB1(118) and ClipR-59 (119)] as well Akt's binding to CTMP (120) and TRB3 (121) that have been reported to favor less Akt phosphorylation.

Acknowledgements

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Table 4.1 Summary of t-tests for CR versus AL at each time and insulin dose.

CR versus AL	Basal			Insulin		
	5 min	15 min	50 min	5 min	15 min	50 min
$pIR^{Tyr1162/1163}$	NS	NS	NS	NS	NS	NS
$pIRS\text{-}1^{Tyr}$	NS	NS	NS	NS	NS	NS
IRS-1-PI3K	NS	NS	NS	NS	NS	NS
$pAKT^{T308}$	NS	NS	NS	NS	NS	P<0.05
$pAKT^{S473}$	NS	NS	NS	NS	NS	P<0.05
2-DG Uptake	ND	ND	ND	ND	ND	P<0.05

Summary of results of student's t-tests compare CR versus AL values for each measurement ($IR^{Tyr1162/1163}$, $IRS\text{-}1^{Tyr}$, IRS-1-PI3K, Akt^{Thr308}, Akt^{Ser473}, and 2-Deoxy-D-glucose uptake) made at each time of insulin exposure (5, 15, and 50 minutes of insulin) and each insulin dose (no insulin, basal, and 1.2 nM insulin). Not significantly different, NS; Not determined, ND. Student's t-test, *P<0.05.

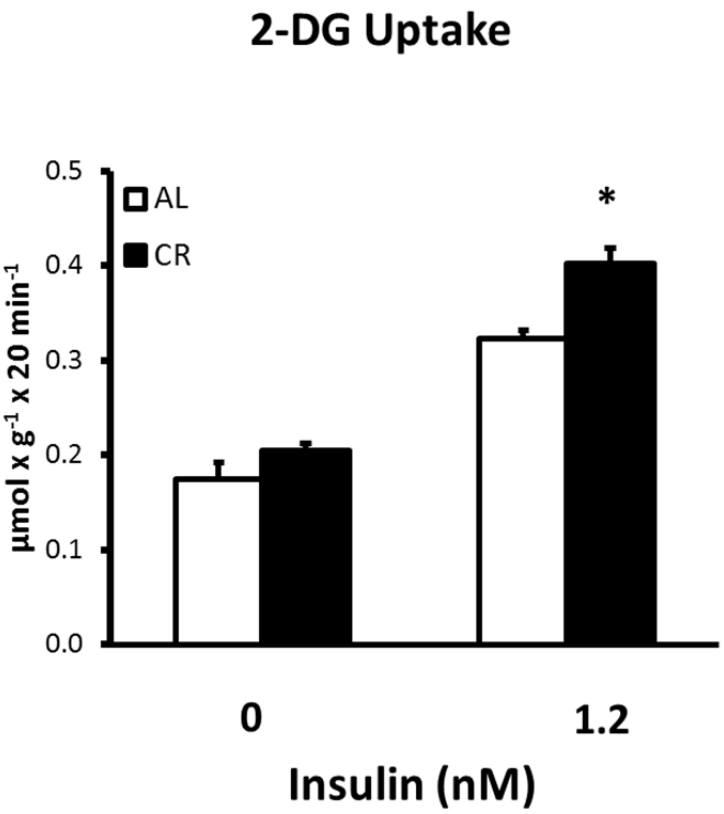


Figure 4.1 2-Deoxy-D-glucose (2-DG) uptake in isolated soleus from adult rats. 2-DG uptake in the soleus with 0 or 1.2nM insulin. Open bars=ad libitum (AL). Closed bars = calorie restriction (CR). *$p<0.05$, CR versus AL in the same insulin treatment group. Data are means ±SEM. N = 6 muscles per diet group and insulin concentration.

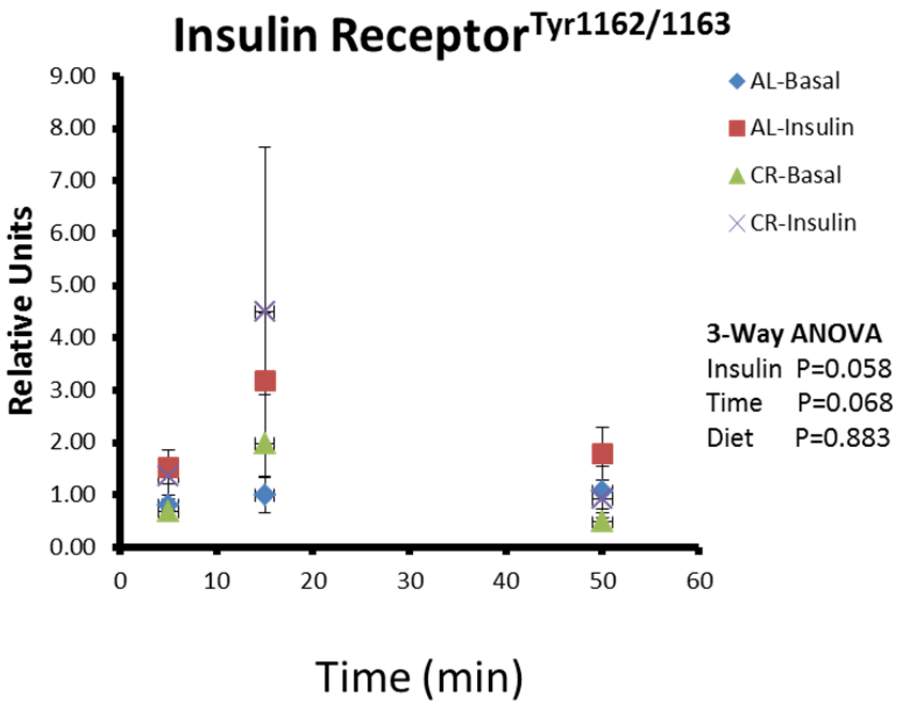


Figure 4.2 Time course of IR$^{Tyr1162/1163}$ phosphorylation. IR$^{Tyr1162/1163}$ phosphorylation in the soleus with 0 or 1.2nM insulin at 5, 15, and 50 minutes. Main effects on diet, insulin and time from 3-way ANOVA are shown. Data are means ±SEM. N=6 muscles per diet group and insulin concentration.

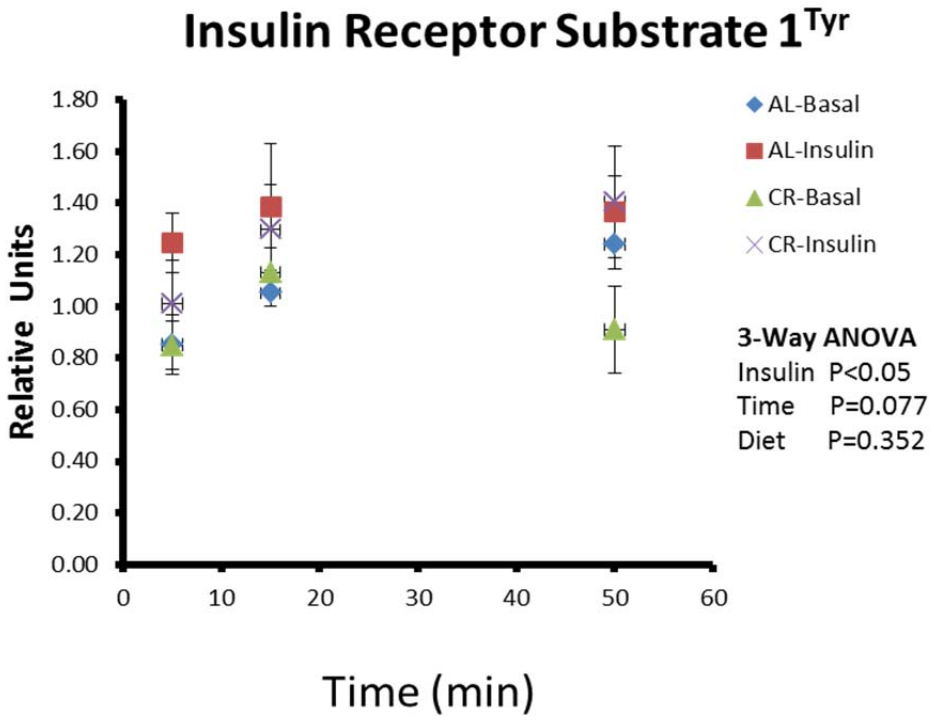


Figure 4.3 Time course of IRS-1^{Tyr} phosphorylation. IRS-1^{Tyr} phosphorylation in the soleus with 0 or 1.2nM insulin at 5, 15, and 50 minutes. Main effects on diet, insulin and time from 3-way ANOVA are shown. Data are means ±SEM. N=6 muscles per diet group and insulin concentration.

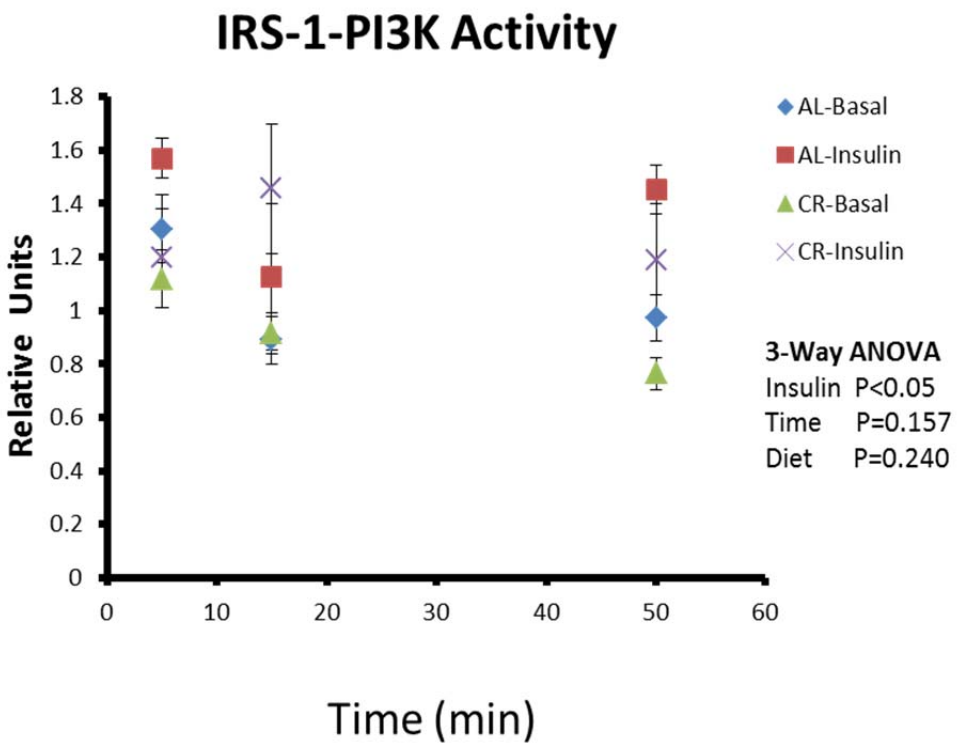
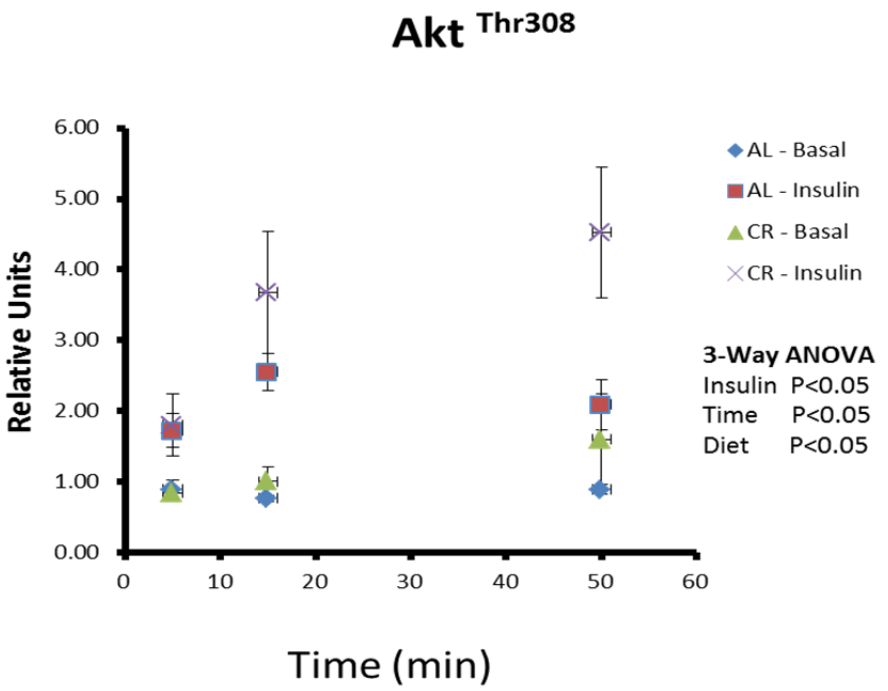


Figure 4.4 Time course of IRS-1-associated PI3K Activity. IRS-1-associated PI3K activity in the soleus with 0 or 1.2nM insulin at 5, 15, and 50 minutes. Main effects on diet, insulin and time from 3-way ANOVA are shown. Data are means ±SEM. N=6 muscles per diet group and insulin concentration.



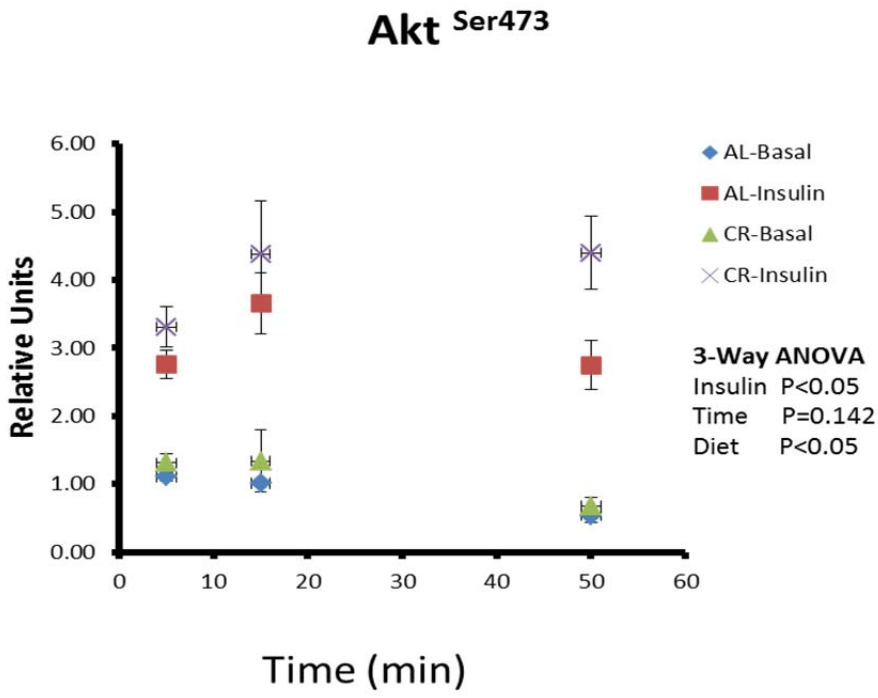


Figure 4.5 Time course of Akt^{Thr308} and Akt^{Ser473} phosphorylation. Akt^{Thr308} and Akt^{Ser473} phosphorylation in the soleus with 0 or 1.2nM insulin at 5, 15, and 50 minutes. Main effects on diet, insulin and time from 3-way ANOVA are shown. Data are means ±SEM. N=6 muscles per diet group and insulin concentration.

CHAPTER V

STUDY 3

In Vivo Assessment of the Effects of Calorie Restriction on Insulin-stimulated Glucose Uptake and the Insulin Signaling Pathway in Multiple Skeletal Muscles

ABSTRACT

The purpose of this study was to evaluate the mechanisms of in vivo insulin-stimulated glucose uptake and phosphorylation of key insulin regulated proteins (insulin receptor, IR; Akt; AS160 and filamin C) in multiple skeletal muscles from adult ad libitum fed and CR (consuming 65% of ad libitum intake) 9mo-old Fisher 344 X Brown Norway rats under euglycemic-hyperinsulinemic clamp conditions. We found that glucose infusion rates were 78% higher ($P<0.001$) in CR (31.7 ± 1.6 mg/kg^{-1} x min^{-1}) versus AL (17.8 ± 0.8 mg/kg^{-1} x min^{-1}) rats with matched plasma insulin levels (AL, 141.3 ± 9.1 μU/ml; CR, 140.3 ± 6.7 μU/ml) thus CR leads to greater whole body insulin sensitivity. We evaluated four different predominantly fast-twitch (epitrochlearis, gastrocnemius, tibialis anterior, and plantaris) and two predominantly slow-twitch (adductor longus and soleus) skeletal muscles and found a significant increase in insulin-

stimulated glucose uptake in the epitrochlearis, gastrocnemius, and tibialis anterior for CR versus AL rats. Phosphorylation of IR was not different between AL and CR rats in any muscle. The most uniform insulin signaling effect of CR compared to AL was an increase in insulin-stimulated Akt phosphorylation at Ser473 and Thr308 in the epitrochlearis, gastrocnemius, tibilais anterior, and soleus muscles. Akt Ser473 phosphorylation alone increased for CR versus AL in the plantaris muscle however there was a strong statistically non-significant trend (P=0.07) for an increase at Akt Thr308. Adductor longus did not differ between AL and CR at either Akt phosphorylation sites. There was not a consistent diet effect on AS160 or filamin C phosphorylation with CR versus AL. The results suggest that CR leads to greater whole body insulin sensitivity attributable to, at least in part, CR effects on in vivo insulin-stimulated glucose uptake by multiple predominantly fast-twitch skeletal muscles, but the CR effects on in vivo insulin-mediated glucose uptake and the associated insulin signaling mechanisms are not identical in all skeletal muscles.

INTRODUCTION

Calorie restriction (CR) without malnutrition (consuming ~60-75% of ad libitum, AL, intake) has been demonstrated to improve whole body insulin sensitivity in various species, including humans (40-42), non-human primates (39), rats (38) and mice (8, 37). Because up to 80% of insulin-stimulated blood glucose clearance is taken up by skeletal muscle (2), it is reasonable to expect that CR leads to increased insulin-mediated glucose uptake in skeletal muscle. Supporting this idea, a number of studies using isolated mouse muscle (8, 37) or isolated (3-5, 9, 10, 21, 23, 24, 85, 87, 122) or perfused rat (53) skeletal

muscle have reported increased insulin-stimulated glucose uptake for CR versus AL animals. Apparently only one publication has evaluated the effects of several months of CR on insulin-stimulated glucose uptake by individual rat skeletal muscles under in vivo conditions. Surprisingly, the results of this study indicated that CR by 8 mo-old rats (consuming ~80% of AL intake beginning at 5 mo-old) did not significantly increase insulin-stimulated glucose uptake of two skeletal muscles of the hind-limb during a euglycemic-hyperinsulinemic clamp (93). However, the same study reported for 24 mo-old rats that CR (consuming ~80% of AL intake beginning at 21 mo-old) resulted in an increased glucose uptake by hind-limb skeletal muscles during a euglycemic-hyperinsulinemic clamp.

The mechanisms that account for improved CR effects on insulin-stimulated glucose uptake have been most frequently studied in skeletal muscle under ex vivo conditions. These studies have documented that when skeletal muscle is exposed to insulin ex vivo, CR can enhance the effect of insulin on selected proteins in the insulin signaling pathway that controls the subcellular distribution of GLUT4, the insulin-regulated glucose transporter protein. Phosphorylation of Akt (also known as protein kinase B, PKB) on both Ser473 and Thr308 is essential for the full effect of insulin on glucose transport (8). In this context, it is notable that the most consistent result of a series of studies on isolated muscles from CR versus AL rodents has been elevated insulin-induced phosphorylation of Akt for isolated muscles from CR rats (9, 10, 24) and mice (8). Akt substrate of 160 kDa (also known as AS160 or TBC1D4) is the most distal substrate of Akt that has been clearly linked to insulin's activation of GLUT4 translocation in skeletal muscle (18, 19). AS160 undergoes Akt-dependent

phosphorylation on several sites, with Thr642 and Ser588 being the sites that appear to account for most of insulin's effect on glucose transport (19). CR was recently reported to lead to greater insulin-mediated AS160 phosphorylation on both Thr642 and Ser588 in isolated epitrochlearis muscle from 9 mo-old rats (24).

Compared to the relatively detailed information about CR effects on insulin signaling in muscle ex vivo, relatively few studies have focused on insulin signaling in rodent muscle of CR rats exposed to insulin in vivo. Furthermore, these studies have focused on the effects of a supraphysiologic bolus injection of insulin rather than on insulin signaling in response to physiologic insulin concentrations (7, 27, 89, 123). A major gap in current knowledge is the lack of any published data on the effect of in vivo administration of a physiologic insulin dose on insulin signaling in skeletal muscle from CR compared to AL rats.

Accordingly, the first major aim of the current study was to compare adult (9 mo-old) CR (60-65% of AL intake initiated at 3 mo-old) versus age-matched AL rats with regard to key insulin signaling steps (insulin receptor tyrosine phosphorylation, phosphorylation of Akt on Ser473 and Thr308, and phosphorylation of AS160 on Ser588 and Thr642) in rat skeletal muscles (epitrochlearis, gastrocnemius, tibialis anterior, plantaris, adductor longus, and soleus) stimulated by a physiologic insulin concentration during a euglycemic-hyperinsulinemic clamp. A second major goal was to measure in vivo glucose uptake in each of these muscles using radiolabeled 2-deoxyglucose (2DG). In addition, whole body insulin sensitivity was assessed based on glucose infusion rate during the clamp, and body composition was determined by a Minispec LF9011 NMR-based analyzer.

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, all chemicals were purchased from Fisher Scientific (Hanover Park, IL) or Sigma Chemical (St. Louis, MO). Reagents and apparatus for SDS-PAGE and immunoblotting were obtained from Bio-Rad Laboratories (Hercules, CA). Anti-phospho-AS160 Ser588 (pAS160Ser588; catalog no. 3028P2) was from B-Bridge International (Mountain View, CA); anti-phospho-Akt Thr308 (pAktThr308; catalog no. 9275), anti-phospho-Akt Ser473 (pAktSer473; catalog no. 9272 and anti-rabbit IgG-horseradish peroxidase conjugate (catalog no. 7074) were from Cell Signaling Technology (Danvers, MA); Anti-phospho-IR Tyr1162/1163 (pIRTyr1162/1163; catalog no. 44-504G) Invitrogen (Camarillo, CA); Anti-phospho-AS160 Thr642 (pAS160Thr642; catalog no. 07-802) and anti-sheep IgG horseradish peroxidase conjugate (catalog no. 12-342) were from Millipore (Billerica, MA). West Dura Extended Duration Substrate (catalog no. 34075) was from Pierce (Rockford, Il). The bicinchoninic acid protein assay kit (catalog no. 23227) was from Thermo Scientific (Rockford, Il).

Animal care. Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. Male Fischer 344 × Brown Norway, F1 generation rats were obtained at 3 mo of age from Harlan (Indianapolis, IN). Animals were housed individually in shoebox cages and maintained on a 12:12-h light-dark cycle (lights out at 1700) in specific pathogen-free conditions. Animals had free access to food (Lab Diet 5053, PMI Nutritional International, Brentwood, MO) and water for a 2-wk acclimation period. Animals then had free access to food (NIH31 chow, Test Diet,

Richmond, IN) and water for another 2-wk acclimation period. During this time, food consumption of all rats was measured daily between 1530 and 1630 to determine baseline food intake (food provided minus food remaining). After the acclimation period, rats were ranked by weight (lowest to highest) and alternately assigned to the AL (ad libitum) group or the CR (calorie restricted) group, so that the initial mean weight was similar for both groups. After the 2-wk acclimation period, the AL group had ad libitum access to the NIH31 chow for the duration of the study. The CR group received NIH31/NIA Fortified chow (Test Diet), which contains extra vitamin supplementation to provide CR animals with a level of vitamins similar to that of animals allowed ad libitum access to the NIH31 diet. The CR group was restricted to 60–65% of the intake of the AL group gradually over 3 wk (90%, 75%, 60–65%). Thereafter, the CR group received 60–65% of the intake of the AL group daily for ~6 mo (182–200 days). All rats were fed between 1530 and 1630 each day, and food intake of both groups was measured daily. All rats were weighed weekly at the same time of day.

Body composition analysis. Body fat, lean mass, and free fluid were measured using an NMR-based analyzer (Minispec LF90II, Bruker Optics, Billerica, MA). The measurements took less than 2 minutes while conscious rats were placed individually in the measuring tube.

Surgical procedures. AL (n=13) and CR (n=14) rats had catheters surgically placed into the jugular vein and carotid artery to sample blood. All surgical instruments were initially autoclaved and sterilized using a hot glass bead sterilizer. Rats were anesthetized

with an intraperitoneal injection of ketamine (50-90 mg/kg) and xylazine (5-10 mg/kg). The ventral neck and back of the head was shaved and the skin was prepared with 3 alternating scrubs of iodine and 70% ethanol. Under aseptic conditions, a small incision was made superior to the clavicle, exposing the carotid artery and jugular vein. Both vessels were catheterized and ligated in place with non-absorbable suture. Catheters were tunneled subcutaneously using a 16 gauge needle and exteriorized at the back of the neck via stainless steel connectors that were coated with medical silicon and fixed subcutaneously upon the closure of the incision. The catheters were filled with heparinized saline and tightly plugged with stainless steel surgical wire. Post-operatively, animals were placed in an isolator equipped with a heating pad for recovery. Ampicillin (100 mg/kg, intravenously) was given and if needed, buprenorphine (0.01-0.5 mg/kg, subcutaneously) for pain every 8-12 h. Warm ringers solution was given to replace lost fluids (1 ml/100 g bw/h surgery).

Procedures for euglycemic hyperinsulinemic clamp (clamp). At approximately 0800 on the morning of clamp experiments, food was removed from rats (~5 h prior to the start of the clamp and infusion procedures). The clamp protocol consisted of a 120 min experimental clamp period (t = 0 to 120 min). At t = -10 min, a blood sample (~100 µl) was taken from the arterial catheter for assessment of basal levels of insulin and glucose. The insulin infusion was begun at t = 0 with a primed-continuous infusion of human insulin (Novo Nordisk). Euglycemia (120-130 mg/dL) was maintained during the clamp by measuring blood glucose every 10 min starting at t = 0 and infusing 50% glucose at variable rates accordingly. Blood insulin concentrations were determined from samples

taken at t = -10 and 120 min. Insulin infusion rates were selected with the goal of achieving similar plasma values for insulin in the CR group compared to the AL group. We have previously reported that CR rats compared to AL rats have higher C-peptide-to-insulin ratio suggesting that insulin clearance is greater for CR animals (22). Others have reported that CR versus AL that were injected with [^{125}I]-insulin have greater hepatic binding of insulin (124, 125). Therefore, to achieve similar plasma insulin in CR and AL rats, insulin was infused at a higher rate for CR rats (4.7 to 6.0 mU·kg^{-1}·min^{-1}) versus AL rats (4.0 mU·kg^{-1}·min^{-1}). The insulin fusion rate for AL rats was 4.0 mU/kg/min, and insulin-infusion rate for CR rats was 4.7 to 6.0 mU/kg/min.

Blood and plasma analysis. Blood glucose during clamps was measured using an Accu-Chek glucometer (Roche, Germany). Plasma insulin was measured using Linco rat/mouse insulin ELISA kits. For determination of plasma radioactivity of [1-^{14}C]2DG, plasma samples were deproteinized with zince sulfate (0.3N) and barium hydroxide (0.3N) and counted using a Liquid Scintillation Counter (Beckman Coulter LS6500 Multi-purpose Scintillation Counter) (126-128).

Skeletal muscle glucose uptake. To estimate glucose uptake in skeletal muscle, a bolus injection of [1-^{14}C]-2-deoxyglucose ([^{14}C]2DG; PerkinElmer) was given at t = 120 min while continuously maintaining the hyperinsulinemic-euglycemic steady-state. At the end of the experiment, skeletal muscles (epitrochlearis , gastrocnemius, tibialis anterior, plantaris, adductor longus, and soleus) were collected and immediately frozen in liquid nitrogen. A portion of each muscle was used for later analysis of muscle [^{14}C]2DG-6-

phosphate ([^{14}C]2DG6P). Muscles used for this analysis were homogenized in 0.5% perchloric acid and centrifuged at 2,000g for 15 min at 4°C. The supernatants were neutralized with potassium hydroxide (5N). An aliquot of the homogenate was quantified by liquid scintillation counting (Perkin Elmer TRI-CARB 2800TR Scintillation Counter) to determine total tissue values (disintegrations per minute,dpm) for the sum of [^{14}C]2DG and [^{14}C]2DGP. Another aliquot was deproteinized with zinc sulfate (0.3N) and barium hydroxide (0.3N) to precipitate the [^{14}C]2DG6P and quantify [^{14}C]2DG in the supernatant. The value for the [^{14}C]2DG in the supernatant (dpm) was subtracted from the total tissue [^{14}C]2DG and [^{14}C]2DGP (dpm) to calculate the glucose uptake rate as indicated by the skeletal muscle [^{14}C]2DGP accumulation. Another aliquot of muscle homogenate was used to determine total protein concentration using the bicinchoninic acid protein assay kit, and the glucose uptake value was expressed relative to muscle total protein concentration (126-128).

Immunoblotting. Equal amounts of protein from each sample were mixed with 6× Laemmli buffer, boiled with SDS loading buffer for 5 min, separated by 10% SDS-PAGE, and then transferred to nitrocellulose. Membranes 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), blocked with 5% bovine serum albumin (BSA) in TBST for 1 h at room temperature and transferred to primary antibody 1:1000 in TBST plus 5% BSA overnight at 4°C. Blots were washed 3 x 5 min with TBST and incubated in buffer containing the appropriate secondary antibody (1:20,000 dilution) for 1 h at room temperature. Membranes were then washed 3 x 5 min with TBST and subjected to enhanced

chemiluminescence for visualization of protein bands. Immunoreactive proteins were quantified by densitometry (AlphaEase FC, Alpha Innotech, San Leandro, CA).

Statistical analyses. Data were analyzed using SigmaPlot, version 11.0 (SPSS, San Jose, CA). Student's t-test was used for comparisons between two groups (i.e., AL versus CR for glucose infusion rate, body mass and body composition measurements). Data are presented as mean ± SEM. A p value ≤ 0.05 was accepted as statistically significant.

RESULTS

Body Mass, Body Composition, Blood Glucose, and Blood Insulin

Body mass was greater ($P < 0.05$) in the AL (417.4 ± 9.4 g) versus CR (274.3 ± 4.3 g) rats. Body fat percentage was greater ($P < 0.05$) for AL (15.4 ± 0.5%) versus CR (7.1 ± 0.6%) rats. Lean body mass percentage was greater ($P < 0.05$) in the CR (78.1 ± 0.4%) versus AL (70.0 ± 1.4%) rats. The fluid percentage was not different between AL (7.6 ± 0.2%) and CR (7.9 ± 0.1%). The fasting blood glucose was not significantly different between AL (106.5 ± 2.7 mg/dl) and CR (100.9 ± 4.24mg/dl) ($P=0.28$). Fasting insulin levels were greater ($P < 0.005$) for AL (53.7 ± 5.6 μU/ml) versus CR (28.3 ± 4.5 μU/ml) rats.

Euglycemic-Hyperinsulinemic Clamp

Glucose levels during the clamp were maintained at 120.7 ± 2.7 mg/dl for AL and 126.1 ± 2.4 mg/dl CR. Insulin levels during the clamp were similar for AL (141.3 ± 9.1 μU/ml) and CR (140.3 ± 6.7 μU/ml). Glucose infusion rates were 78% higher ($P<0.001$) in CR (31.7 ± 1.6 $mg/kg^{-1} \times min^{-1}$) versus AL (17.8 ± 0.8 $mg/kg^{-1} \times min^{-1}$) rats.

2-Deoxy-D-glucose Uptake

Epitrochlearis 2-DG uptake was significantly greater ($P < 0.05$) for CR versus AL rats. In the gastrocnemius, 2-DG uptake increased ($P < 0.05$) in CR versus AL rats. Also in the tibialis anterior 2-DG uptake was significantly increased ($P < 0.05$) for CR versus AL rats. There were no significant diet effects for 2-DG uptake between AL and CR rats in the plantaris, adductor longus, or soleus muscles. (Figure 5.1)

Insulin Receptor Phosphorylation

There were no significant diet effects on IR (insulin receptor) Tyr1162/1163 phosphorylation between AL and CR rats in the epitrochlearis, gastrocnemius, tibalis anterior, plantaris, adductor longus, or soleus (Figure 5.2).

Akt Phosphorylation

In the epitrochlearis, Akt phosphorylation increased in CR versus AL rats at both the Thr308 ($P<0.05$) and Ser473 sites ($P<0.05$). For the gastrocnemius, Akt phosphorylation for CR versus AL rats was increased at the Ser473 site ($P<0.05$) and increased at the T308 site ($P=0.05$) with CR versus AL rats. There were significant increases in Akt phosphorylation in the CR versus AL rats in the tibialis anterior at both Thr308 ($P=0.05$) and Ser473 ($P<0.05$). In the plantaris, Akt phosphorylation increased in CR versus AL at the Ser473 site ($P<0.05$) and tended to increase at the Thr308 site ($P=0.07$). There were no significant diet effects on Akt phosphorylation at either site in

the adductor longus muscle. In the soleus, Akt phosphorylation increased for CR versus AL at both the Thr308 ($P<0.05$) and Ser473 ($P<0.05$) sites (Figure 5.3 and 5.4).

AS160 Phosphorylation

In the epitrochlearis of CR versus AL rats, AS160 phosphorylation tended to increase at the Thr642 site ($P=0.08$) and increased at the Ser588 site ($P<0.05$). In the gastrocnemius, AS160 phosphorylation at the Thr642 site or the Ser588 site did not change significantly with diet. In the tibialis anterior of CR versus AL rats, AS160 phosphorylation increased at both the Thr642 ($P<0.05$) and Ser588 ($P<0.05$) sites. In the plantaris, AS160 phosphorylation did not increase at the Thr642 site and tended to increase ($P=0.06$) at the Ser588 site. In the adductor longus, AS160 phosphorylation at the Thr642 site or the Ser588 site did not change significantly with diet. In the soleus, AS160 phosphorylation increased at the Thr642 site ($P<0.05$) and did not change at the Ser588 site (Figure 5.5 and 5.6).

Filamin C Phosphorylation

In the plantaris of CR versus AL rats, filamin C phosphorylation at the Ser2213 site increased ($P<0.05$). There were no significant diet effects on filamin C Ser2213 phosphorylation in the epitrochlearis, gastrocnemius, tibalis anterior, adductor longus, or soleus (Figure 5.7).

DISCUSSION

Our research group has previously shown that CR can enhance insulin-stimulated glucose uptake in skeletal muscle under ex vivo conditions (3, 8-10, 21, 24, 25, 85-87, 114). The current study is the first to look at the effects of CR on in vivo glucose uptake by skeletal muscle together with the associated insulin signaling events. We determined glucose uptake and phosphorylation of key insulin signaling proteins in AL and CR rats with insulin levels elevated to similar physiologic levels in both diet groups. We evaluated four different predominantly fast-twitch (epitrochlearis, gastrocnemius, tibialis anterior, and plantaris) and two predominantly slow-twitch (adductor longus and soleus) skeletal muscles (81) and found a significant increase in insulin-stimulated glucose uptake in three out of six skeletal muscles studied. There was increased 2-DG uptake in the epitrochlearis, gastrocnemius, and tibialis anterior for CR versus AL rats and no significant difference between diet groups for plantaris, adductor longus, and soleus. We evaluated the most proximal insulin signaling protein, the insulin receptor (IR). Phosphorylation of IR was not different between AL and CR rats. The most consistent effect of CR compared to AL was a robust increase in insulin-stimulated Akt phosphorylation. We looked at the two important sites on Akt (Ser473 and Thr308) that are phosphorylated upon activation by insulin, and we found an increase with CR versus AL at both sites in the epitrochlearis, gastrocnemius, tibilais anterior, and soleus muscles. Akt Ser473 phosphorylation alone increased for CR versus AL in the plantaris muscle however there was a strong statistically non-significant trend (P=0.07) for an increase at Akt Thr308. Adductor longus did not differ between AL and CR at either Akt phosphorylation sites. AS160 is an Akt substrate and the most distal insulin signaling protein that is linked to insulin-stimulated glucose uptake in skeletal muscle. We found

that AS160 (Thr642 and Ser588) phosphorylation was increased with CR versus AL in the epitrochlearis and tibialis anterior. AS160 phosphorylation increased only at Thr642 in the soleus muscle and in the plantaris had a statistically non-significant trend to increase only at Ser588 (P=0.06). We found no evidence for an increased AS160 phosphorylation in the gastrocnemius or adductor longus. Filamin C is a highly expressed protein in skeletal muscle (129) and believed to be involved with stabilizing actin filament structures (130). Actin filaments have been implicated to play a role with the insulin-mediated regulation of GLUT4 transporter vesicle movement (131) but the possible functional significance of filamin C for GLUT4 translocation has not been tested. Filamin C was previously identified as an Akt substrate that is phosphorylated on Ser2213 in insulin-stimulated C2C12 myocytes (129). Interestingly, it has been demonstrated that the CR-related increase in activation of Akt is accompanied by greater phosphorylation of filamin C on Ser2213 in rat skeletal muscle (Sharma, Arias, Sequea and Cartee, unpublished results). In the current study, in vivo filamin C phosphorylation on Ser2213 increased with CR versus AL only in the plantaris muscle.

Using the euglycemic-hyperinsulinemic clamp, we demonstrated that overall insulin sensitivity is increased with calorie restriction in the FBN rat. In a previous publication, Escriva et al. (93) also found that rats that were calorie restricted had higher glucose disposal rates indicating that calorie restriction increased whole body insulin sensitivity. Earlier studies using the euglycemic clamp had also found calorie restriction can improve whole body insulin sensitivity in rhesus monkeys (36, 39) and humans (40-42).

Escriva et al. also measured muscle-specific glucose disposal in the soleus and quadriceps femoris from 8 mo-old rats and reported that CR did not significantly increase in vivo insulin-stimulated glucose uptake in either of these muscles. The results for the soleus are reminiscent of the current study where we found that glucose uptake was also not increased in the soleus. We also found no evidence for CR to increase glucose uptake by the adductor longus, which has been reported to have a fiber type composition (90% type I and 10% type II) similar to the soleus (88% type I and 12% type II) (81). The quadriceps femoris is a predominantly fast-twitch muscle (2% type I and 98% type II). In our study we found a significant CR versus AL effect in glucose uptake in three predominantly fast-twitch muscles: the epitrochlearis (8% type I and 92% type II), gastrocnemius (10% type I and 90% type II), and tibialis anterior (1% type I and 99% type II) muscles, but not in the predominantly fast-twitch plantaris (4% type I and 96% type II). It is not clear why there was no significant CR result in the quadriceps femoris and plantaris muscles compared to the significant CR effect on 3 of 4 predominantly fast-twitch muscle in the current study, but it is possible that a similar reason is relevant for the lack of a detectable CR effect on the quadriceps and the plantaris. Other important differences between the experiment by Escriva et al. versus the current study include: difference in strain (Wistar versus Fischer Brown Norway), the duration (3 months versus 6 months) and degree (80% versus 65% of AL intake) of calorie restriction, or differences in the clamp procedures. The euglycemic-hyperinsulinemic clamp of Escriva et al. was conducted with anesthetized rats in which the surgery was performed immediately prior to the clamp whereas in the current study rats were conscious throughout the clamp procedure and the surgery was performed 7 days prior to the clamp.

In spite of these differences in experimental design and methods, the similarity between the key results for CR effects on glucose uptake in 8-9 mo-old rats is notable (greater whole body insulin sensitivity, lack of a significant effect on the soleus, and the lack of a significant effect on at least one predominantly fast-twitch muscle).

Although there is very limited literature regarding the effect of CR on glucose uptake in rodents in skeletal muscles in vivo, many previous studies have documented CR-induced increases in insulin-stimulated glucose uptake by isolated skeletal muscle ex vivo (3, 8-10, 21, 24, 25, 85-87, 114). A recent study from our group evaluated ex vivo glucose uptake by epitrochlearis and soleus muscles from rats that undergone the same treatment as the current study. In isolated skeletal muscle, male 9-mo old FBN rats with the same feeding protocol as the rats used in the current study, showed epitrochlearis (predominantly fast-twitch) from CR versus AL rats result in enhanced glucose uptake. In this study the predominantly fast-twitch epitrochlearis muscle showed an increase in glucose uptake for CR versus AL. The isolated soleus (predominantly slow-twitch) muscle from CR versus AL rats had enhanced glucose uptake, however in this study the soleus did have a significant increase for insulin-mediated glucose uptake with CR versus AL. It is notable that in the ex vivo experiment the epitrochlearis had a relatively higher increase in glucose uptake with CR compared to the soleus muscle (61% versus 40%) (24). Although there was not a significant CR effect on in vivo glucose uptake in the soleus, there was a non-significant trend (P=0.26) for 19% greater values for the CR versus AL group. Escriva et al. (93) also found a non-significant trend for ~48% greater glucose uptake by the soleus of CR compared to rats. The lack of a significant CR effect on glucose uptake in the soleus may be explained by a difference in ex vivo versus in

vivo conditions. Differences between the in vivo and ex vivo condition that may influence glucose uptake include but are not limited to: 1) the influence of blood flow, 2) presence of circulating hormones and cytokines (i.e. adiponectin, resistin, leptin, cortiscosterone, etc.) 3) circulating fuels (free fatty acids, amino acids and glucose), and 4) neural recruitment and contractile activity of skeletal muscles. Skeletal muscle glucose uptake, in addition to intrinsic mechanisms in the muscle itself, is regulated by extrinsic factors such as blood flow.

An increase in blood flow delivers more glucose and insulin to skeletal muscle which increases the insulin-stimulated glucose uptake (132). Calorie restriction of obese humans has been shown to increase skeletal muscle blood flow with an insulin infusion (133). In obese, insulin resistant OLETF rats, CR for 8 weeks led to improved whole body insulin sensitivity without detectable effects on insulin-induced vasodilation of isolated microvascular preparations from the red or white gastrocnemius muscles (134). We are not aware of any evidence that muscle blood flow is reduced by calorie restriction in rats, and seem an unlikely explanation of why there was not a significant CR effect on insulin-stimulated glucose uptake in the soleus in vivo. Circulating blood cytokines have been shown to influence insulin sensitivity and may affect in vivo insulin-mediated glucose uptake. Adiponectin and leptin have been shown to increase insulin sensitivity and conversely resistin has been shown to decrease insulin sensitivity (135). In 8 mo-old rats, fasting plasma adiponectin was shown to increase significantly while plasma leptin and plasma resistin significantly decreased in CR versus AL rats (93). Increased adiponectin and decreased resistin would favor increased insulin sensitivity and increased insulin-stimulated glucose uptake and thus would not adequately explain the soleus

muscle result. However, decreased circulating leptin levels is a plausible factor that would favor reduced insulin sensitivity and subsequent insulin-stimulated glucose uptake thus blunting insulin-stimulated uptake in the soleus in vivo relative to ex vivo conditions. Physiological concentrations of corticosterone was shown to have no effect on insulin-stimulated glucose uptake and only supraphysiological levels of corticosterone decreased 2DG uptake in soleus and extensor digitorum longus muscles (136). Corticosterone has been shown to increase with CR versus AL in rats (137, 138), but physiologic levels of corticosterone would not be expected to alter the in vivo insulin-stimulated uptake in the soleus.

Philip Randle described the reciprocal relationship between the oxidation rates of lipids and carbohydrates (called the Glucose Fatty Acid Cycle), and demonstrated that this relationship can be influenced by extracellular concentrations of free fatty acids (139). Plasma free fatty acids have been shown to decrease with CR in old rats during a clamp (140). If plasma free fatty acids were lower in CR rats, it would not be expected to reduce glucose uptake in muscle.

In this study, we reported that fasting plasma glucose was not significantly different between CR and AL rats and a similar observation was also reported previously (93) in another study. High rates of glucose uptake by muscle during the clamp could lead to an increase conversion of glucose-6-phosphate (G-6-P) if hexokinase could not keep pace with the high glucose entry into the muscle via the greater insulin-stimulated translocation of the GLUT4 transporter that has been reported for muscles of CR rats (3). Accumulation of G-6-P can exert end-product inhibition on hexokinase leading to accumulation of free glucose within the muscle which would subsequently result in

counter-transport of glucose and decreased net glucose uptake measured in vivo relative to ex vivo in the absence of glucose. Although plasma glucose was matched between diet groups during the clamp, glucose was not included during the 50 minutes of ex vivo incubation of muscles. When the glucose analog that was present ex vivo (2-deoxyglucose, 2-DG) is phosphorylated to 2-DG-6-P, unlike G-6-P, it is not a potent inhibitor of hexokinase (110). It should also be noted that the measurement of glucose uptake in vivo versus ex vivo was not identical. Ex vivo glucose uptake measurements included total 2-DG and 2-DG-6-P as described previously (110, 141), whereas in vivo glucose uptake measured the accumulation of 2-DG-6-P only as described above. Measuring only 2-DG-6-P would be expected to be lower than 2DG plus 2-DG-6-P if hexokinase were rate-limiting.

Skeletal muscle in vivo maintains physiological neural inputs and recruitment patterns during the course of experimentation. The soleus muscle is a predominantly slow-twitch, postural muscle and is likely recruited for contraction while the conscious rat is standing during the clamp procedure in this study. Increased contraction would seem to favor an insulin-independent increase in skeletal muscle glucose uptake and would increase the in vivo glucose uptake observed in the soleus. Some studies have suggested small differences in spontaneous physical activity of CR versus AL rats, with the greatest differences evident near the time when CR rats are fed (142). Physical activity was not systematically monitored during the clamps, but neither group was noticed to have high levels of activity, and there were not obvious differences between groups. There does not appear to be a compelling reason to expect that in vivo

recruitment of the soleus muscle would likely explain why there was no significant increased insulin-mediated glucose uptake in vivo when compared to the soleus ex vivo.

Other notable differences between the in vivo and ex vivo conditions in our experiment are the dosage and duration of insulin stimulation. The insulin concentration in vivo (~140 μU/ml) versus ex vivo (200 μU/ml) was similar but not identical. We do not expect this small difference in insulin concentration to play a significant role in the skeletal muscle glucose accumulation but it is possible the lower insulin dosage in vivo could have accounted for the lower insulin-stimulated glucose uptake in the soleus in vivo relative to ex vivo. The duration of insulin stimulation during the clamp was approximately 120 minutes compared to 50 minutes with ex vivo incubations. It is possible that the longer exposure of insulin may have caused inhibition via the insulin signaling pathway in vivo versus ex vivo, which would favor decreased insulin-stimulated glucose uptake as was seen in the soleus in vivo versus ex vivo.

Taking together results for CR effects on insulin-stimulated glucose uptake in skeletal muscle under both in vivo and ex vivo are powerful because in combination these methods provide greater insights into the mechanisms by which CR increases insulin sensitivity in skeletal muscle than would not be evident using only one of the approaches. Ex vivo incubations allow greater experimental control for investigating the intrinsic ability of the muscle to respond to insulin and CR without the influence of blood flow, neural inputs, and blood-borne chemokines. In vivo experimentation is advantageous because insulin-stimulated glucose uptake measurements are taken in a physiological context. The influence of usual-living insulin levels, blood flow, neural inputs, and blood chemokines may influence CR's effects on skeletal muscle. In combination, the results

from ex vivo and in vivo experimentation give us the most comprehensive view of the CR effects on insulin-stimulated glucose uptake in skeletal muscle to date.

Phosphorylation of IR was not different between AL and CR rats. This result is consistent with a number of prior experiments examining the phosphorylation state of the IR with CR versus AL with physiologic insulin. In studies analyzing ex vivo CR effect in rats, there was also no difference in insulin receptor phosphorylation between AL and CR muscles incubated with a physiologic insulin dose (24, 114). Published studies that have previously reported increased insulin receptor phosphorylation in vivo with CR used a supraphysiologic dose of insulin via a bolus injection (6, 27).

To our knowledge Akt phosphorylation with CR has not been studied with physiologic insulin in a clamp study. The CR-mediated increase for Akt phosphorylation is very similar to results ex vivo with similar aged rats fed with the exact protocol as the current study. Sharma et al. (24) found that CR significantly increased Akt phosphorylation versus AL fed rats at both the Thr308 and Ser473 sites. Other studies have also seen similar increases in CR for Akt phosphorylation in rats ex vivo (9, 10, 114). In the current study, Akt phosphorylation was increased in the epitrochlearis, gastrocnemius, tibialis anterior, plantaris, and soleus muscles, with only the adductor longus not having a significant increase. Of all of the insulin signaling measurements made in this and previous studies, Akt phosphorylation has been most consistently increased with CR versus AL animals, both in vivo and ex vivo.

There are no other reports on phosphorylation of AS160 with calorie restriction on insulin-stimulated muscles in vivo. In 9 month old rat epitrochlearis muscle incubated ex vivo with physiological insulin there was an increase in the phosphorylation of AS160

at both Thr642 and Ser588 sites. Three out of four (epitrochlearis, tibialis anterior, and plantaris) predominantly fast-twitch muscles in the current study increased in at least one AS160 phosphorylation site. In 9 month old rat soleus muscle incubated with physiological insulin there was no increase in the phosphorylation of AS160 with CR versus AL, and in this study, the soleus muscle had increased phosphorylation at Thr642 but not Ser588 with CR versus AL. In another study of isolated epitrochlearis and soleus muscle from 24 mo-old rats incubated in physiological insulin there was no increase in AS160 phosphorylation with CR versus AL in either muscle (114). In the current study, there was also no increase for AS160 phosphorylation in either the adductor longus or gastrocnemius. In summary, although the CR effect on AS160 phosphorylation appeared to be generally similar for the epitrochlearis of 9 month-old rats under both in vivo and ex vivo conditions, greater AS160 phosphorylation with CR versus AL was not uniformly found in all muscles in either condition.

In 9 month old rat epitrochlearis muscle incubated with physiological insulin there was an increase in the phosphorylation of filamin C on Ser2213 (Sharma et al, unpublished results). Also in 24 month-old rat epitrochlearis and soleus muscle incubated with physiological insulin there was an increase in the phosphorylation of filamin C on Ser2213 (Sequea, Sharma, Arias and Cartee, unpublished results). The current study was the first to report CR effects on filamin C phosphorylation on Ser2213 in muscle collected after in vivo insulin treatment. The only significant increase for CR versus AL was in the plantaris muscle. The current data indicate that, in contrast to results for muscles studied ex vivo, greater filamin C phosphorylation is not a typical result for CR versus AL muscles stimulated with insulin in vivo.

Taking together the results for glucose uptake and insulin signaling in five different muscles offers an opportunity to probe for insights into possible mechanisms for CR-related benefits on insulin sensitivity in muscle. For CR versus AL rats, in vivo glucose uptake was significantly greater in the predominantly fast-twitch epitrochelaris, gastrocnemius, and tibialis anterior muscle. In each of these muscles, the CR-mediated increase in glucose uptake occurred independent of a diet effect on insulin receptor activation together with an increase in Akt phosphorylation. A number of previous studies have consistently reported a CR-associated increase in muscle Akt phosphorylation concomitant with greater insulin-stimulated glucose uptake (8-10, 24, 114). However, in the current study, the CR-related increases in Akt phosphorylation in the soleus and plantaris muscles were not accompanied by significantly increased glucose uptake. Neither Akt phosphorylation nor glucose uptake was significantly increased in the adductor longus muscle of CR versus AL rats. Taken together, these results suggest that the CR-induced increase in Akt activation by greater phosphorylation on Ser473 and Thr308 may be necessary, but not sufficient for greater in vivo insulin-stimulated glucose uptake of CR versus AL rats. There is not a simple relationship between CR effects on glucose uptake and AS160 phosphorylation. For two (epitrochlearis and tibialis anterior) of the three muscles characterized by greater in vivo glucose uptake in CR compared to AL rats, AS160 phosphorylation on at least one site was also greater for CR rats. However, there was not a consistent relationship between CR effects on AS160 phosphorylation and in vivo glucose uptake in the gastrocnemius (only glucose uptake increased with CR) or the soleus (only phosphorylation of Ser588 increased with CR). Under ex vivo conditions in 9 month-old rats, both the epitrochlearis and soleus were

characterized by CR-related improvements in glucose uptake, but only in the epitrochlearis was AS160 phosphorylation greater for CR versus AL rats. Taking together the ex vivo results from previous research and the in vivo results from the current study, it is apparent that CR effects on insulin-stimulated glucose uptake in the soleus are not attributable to CR effects on AS160 phosphorylation on Thr642 or Ser588. There was no correspondence between CR effects on in vivo glucose uptake and filamin C phosphorylation. Based on the current results, the strongest link between CR effects on insulin signaling and glucose uptake are found with greater Akt phosphorylation that is usually observed for CR compared to AL muscles. However, the important intermediate steps between CR effects on Akt and glucose uptake are uncertain. Regulation of in vivo glucose uptake by AS160 and filamin C may be regulated on other Akt-phosphorylation sites not measured in this study. There may be CR effects on AS160 or filamin C that are localized to certain subcellular regions and not evident when analyzing the whole muscle lysate. It is also possible that other unknown Akt substrates may play a role in regulating CR's effect on glucose uptake. It also remains possible that CR can influence glucose uptake by Akt-independent mechanisms.

Regardless of the possible role of different insulin signaling steps for CR effects on glucose uptake, another important issue is to understand how CR effects on the different insulin signaling steps are related to each other. There was no effect of CR on the insulin receptor tyrosine phosphorylation on 1162/1163 observed in any muscle, so other mechanisms must account for the CR effects on post-receptor signaling. We reported that Akt phosphorylation was increased with CR in five out of six muscles and was the most consistent effect of CR. The increase in Akt phosphorylation did not appear

to be a result of increased upstream insulin receptor activation. We did not find a uniform predicative relationship between Akt phosphorylation and AS160 phosphorylation with CR versus AL. Only in the tibialis anterior did phosphorylation on both Akt sites lead to phosphorylation on both AS160 phosphorylation sites. There was increased AS160 phosphorylation in the epitrochlearis, plantaris, and soleus on only one out of two phosphorylation sites despite an increase for both Thr308 and Ser473 on Akt. The adductor longus muscle consistently lacked a diet effect for CR versus AL for the insulin signaling molecules in which we studied. AS160 phosphorylation with CR versus AL was not increased seemingly because there was no increase in the upstream Akt phosphorylation in the adductor longus muscle. It is not clear why the gastrocnemius did not have an increase for AS160 phosphorylation despite an increase in Akt phosphorylation. Similarly, there were no significant diet effects for Ser2213 phosphorylation on filamin C in the epitrochlearis, gastrocnemius, tibialis anterior, or solelus despite an increase in Akt phosphorylation in these muscles. Only in the plantaris muscle did an increase in Akt phosphorylation coincide with an increase in filamin C phosphorylation. The ability of a kinase to phosphorylate substrates depends heavily on proximity. In some cases, the subcellular localization of AS160 and filamin C may not be accessible for activated Akt and may explain why we found a diet effect on Akt but not known Akt substrates in some muscles. In addition, although an increase for total Akt phosphorylation does not lead to an increase for AS160 and filamin C phosphorylation, the Akt2 specific isoform may be a better predictor of AS160 and/or filamin C phosphorylation.

In conclusion, the current data demonstrate that CR leads to greater whole body insulin sensitivity that is, at least in part, attributable to substantial CR effects on in vivo insulin-stimulated glucose uptake by multiple predominantly fast-twitch skeletal muscles. The current results also clearly demonstrate that CR does not uniformly enhance insulin-stimulated glucose uptake in all skeletal muscles. Although CR did not significantly enhance in vivo glucose uptake in either predominantly slow-twitch muscle that was studied, there was also no evidence for a CR effect on glucose uptake by the predominantly fast-twitch plantaris. The results support the idea that CR effects on glucose uptake are, at least in part, related to enhancing Akt phosphorylation. However, the current results indicate that CR effects on neither AS160 (Thr642 and Ser588) phosphorylation nor filamin C Ser2213 phosphorylation are essential for greater glucose uptake in skeletal muscle. This result implies that the signaling mechanisms for CR enhanced insulin-mediated glucose uptake are not identical in all skeletal muscles and increased Akt phosphorylation does not simply translate to increase phosphorylation of AS160 and filamin C. Future studies will be necessary to fully determine the precise role of CR on in vivo glucose uptake and the insulin signaling mechanisms in skeletal muscle. Assessing the effect of CR on Akt-binding proteins in vivo may provide insight into how Akt may be regulated with CR. Further, identifying other insulin-regulated Akt phosphorylation sites on AS160, filamin C, and other known Akt substrates (e.g. TBC1D1 and CDP-138) may elucidate the links between CR effects on Akt phosphorylation and the CR-mediated increase in skeletal muscle glucose uptake.

Acknowledgements

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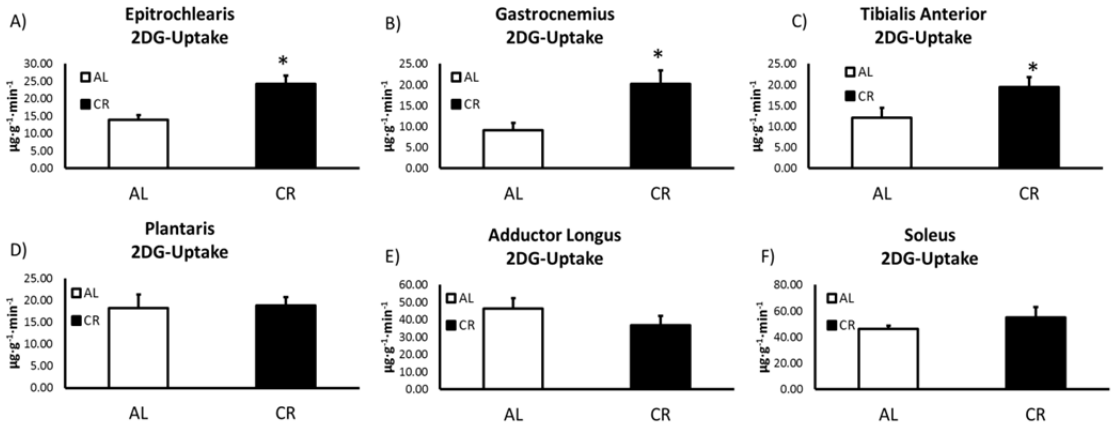

Figure 5.1 In Vivo 2-Deoxy-D-glucose (2-DG) uptake. 2-DG uptake in epitrochlearis (A), gastrocnemius (B), tibialis anterior (C), plantaris (D), adductor longus (E), and soleus (F) muscles in AL and CR treated groups. *$p<0.05$, CR versus AL. Data are means ± SEM. n=6-13 muscles per diet group.

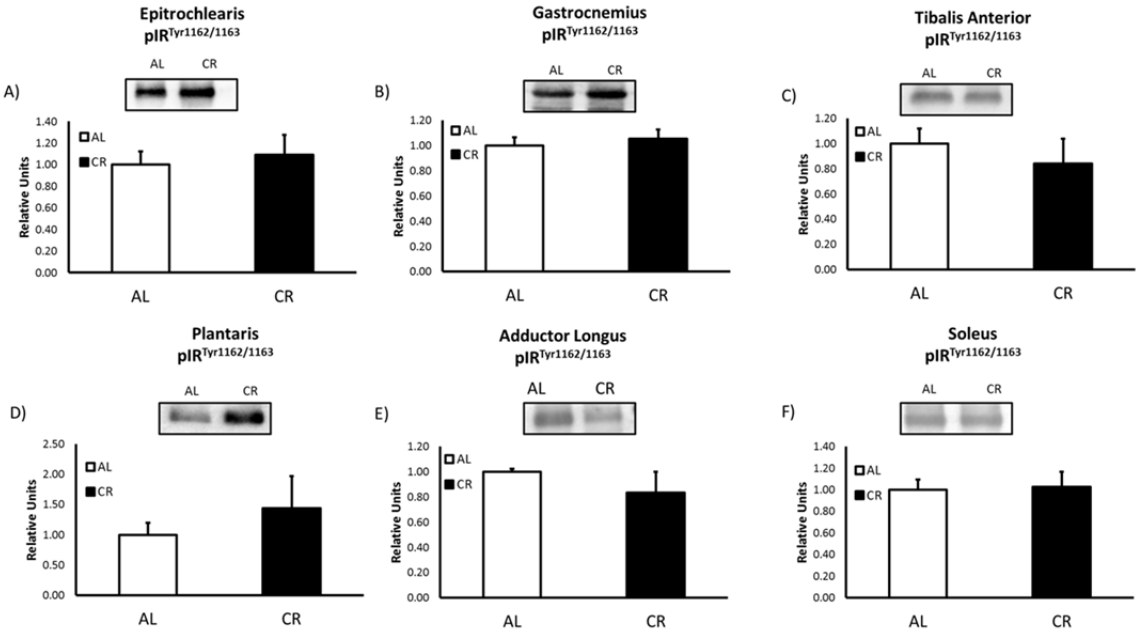

Figure 5.2 In vivo insulin receptor tyrosine phosphorylation (IR$^{Tyr1162/1163}$). IR$^{Tyr1162/1163}$ phosphorylation in epitrochlearis (A), gastrocnemius (B), tibialis anterior (C), plantaris (D), adductor longus (E), and soleus (F) muscles in AL and CR treated groups. *$p<0.05$, CR versus AL. Data are means ± SEM. n=6-8 muscles per diet group.

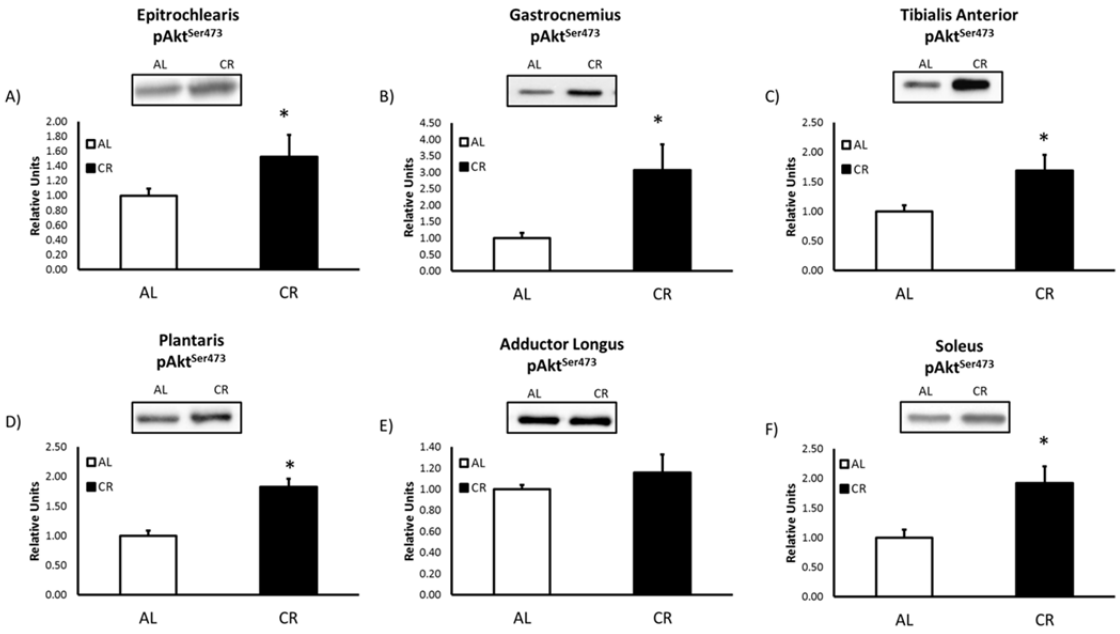

Figure 5.3 In vivo Akt^{Ser473} phosphorylation. Akt^{Ser473} phosphorylation in epitrochlearis (A), gastrocnemius (B), tibialis anterior (C), plantaris (D), adductor longus (E), and soleus (F) muscles in AL and CR treated groups. *$p<0.05$, CR versus AL. Data are means ± SEM. n=6-8 muscles per diet group.

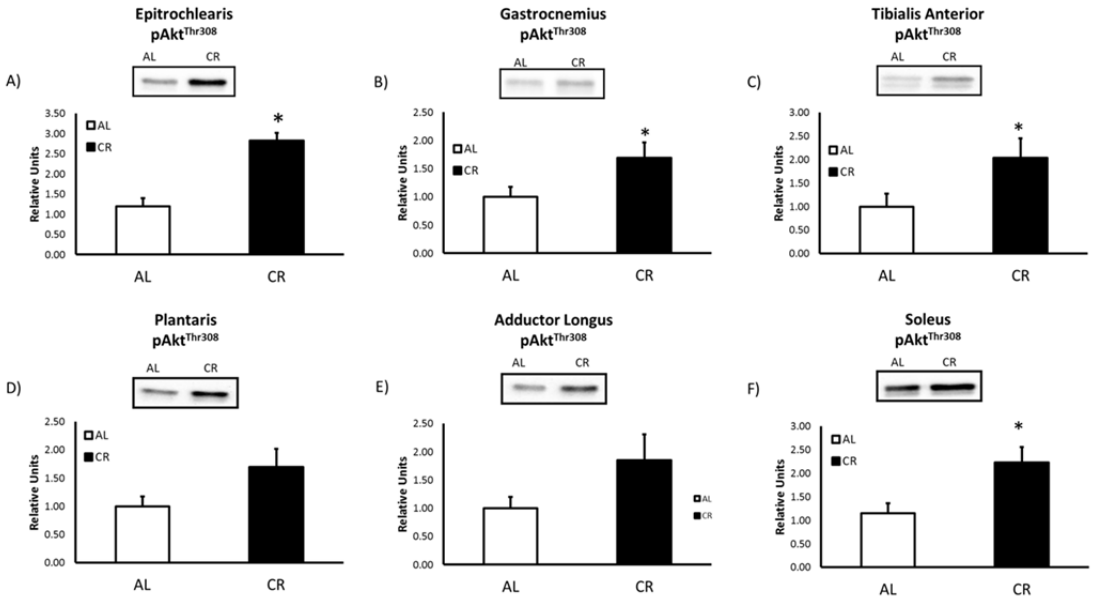

Figure 5.4 In vivo Akt^{Thr308} phosphorylation. Akt^{Thr308} phosphorylation in epitrochlearis (A), gastrocnemius (B), tibialis anterior (C), plantaris (D), adductor longus (E), and soleus (F) muscles in AL and CR treated groups. *$p<0.05$, CR versus AL. Data are means ± SEM. n=5-8 muscles per diet group.

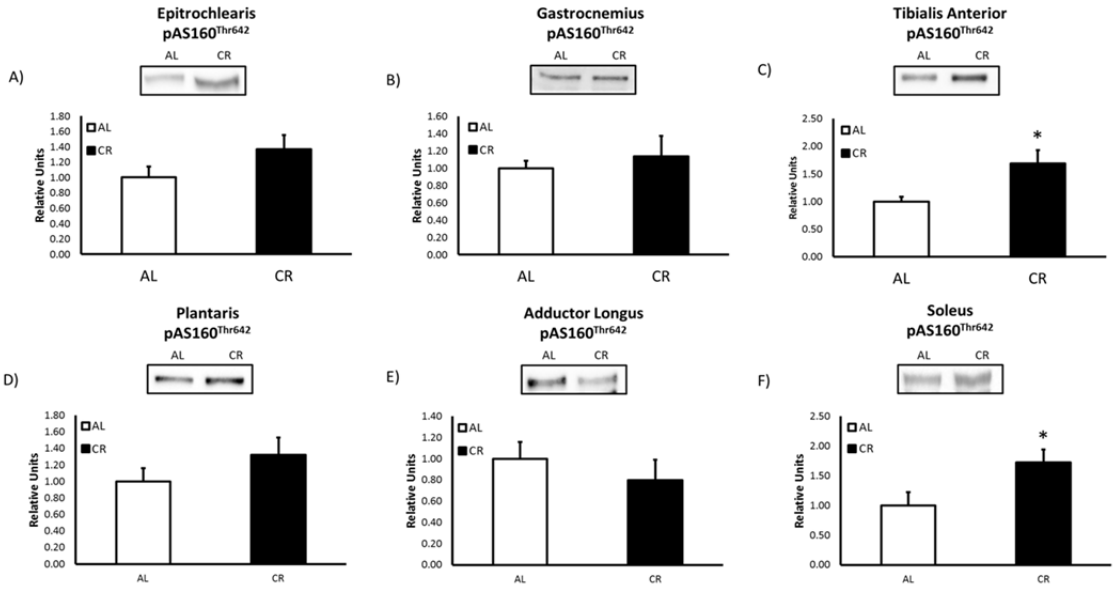

Figure 5.5 In vivo AS160^{Thr642} phosphorylation. AS160^{Thr642} phosphorylation in epitrochlearis (A), gastrocnemius (B), tibialis anterior (C), plantaris (D), adductor longus (E), and soleus (F) muscles in AL and CR treated groups. *$p<0.05$, CR versus AL. Data are means ± SEM. n=6-8 muscles per diet group.

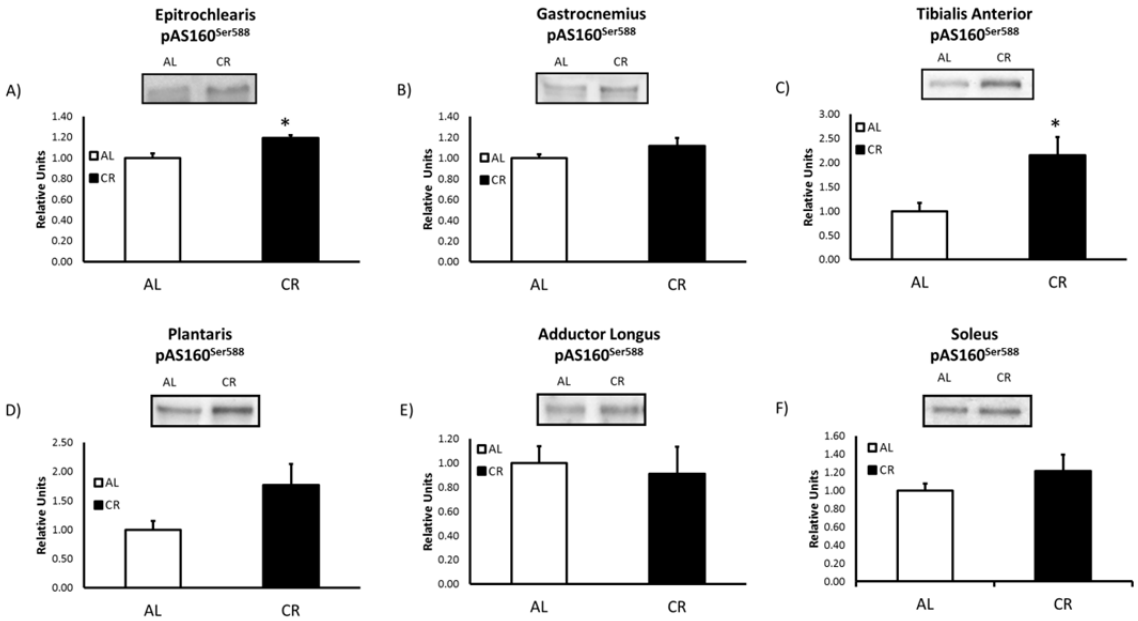

Figure 5.6 In vivo AS160^{Ser588} phosphorylation. AS160^{Ser588} phosphorylation in epitrochlearis (A), gastrocnemius (B), tibialis anterior(C), plantaris (D), adductor longus (E), and soleus (F) muscles in AL and CR treated groups. *$p<0.05$, CR versus AL. Data are means ± SEM. n=5-8 muscles per diet group.

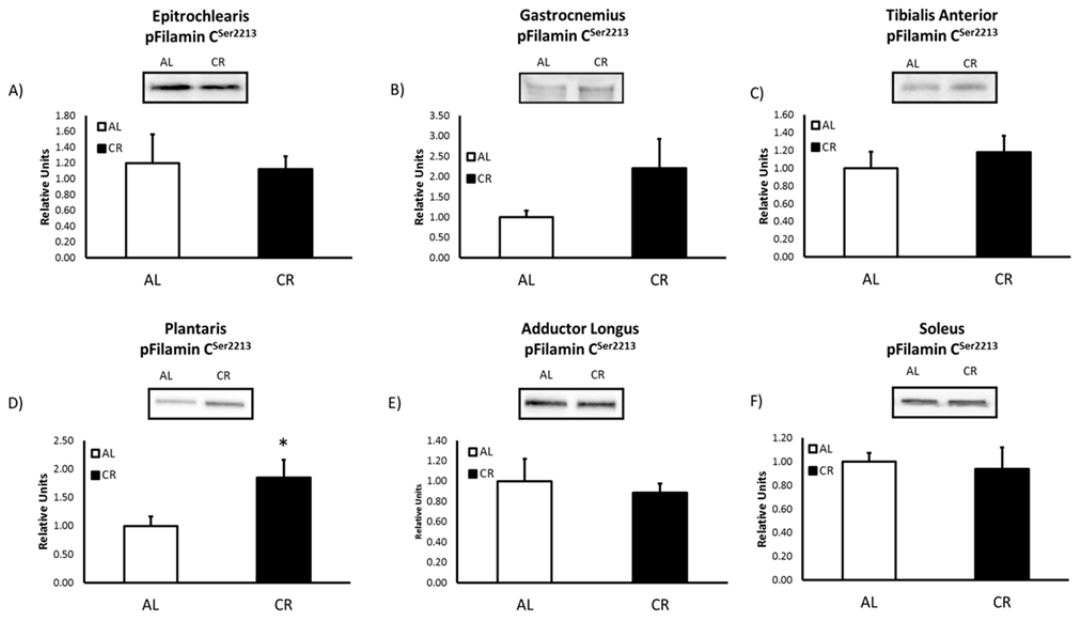

Figure 5.7 In vivo Filamin C^{Ser2213} phosphorylation. Filamin C^{Ser2213} phosphorylation in epitrochlearis (A), gastrocnemius (B), tibialis anterior(C), plantaris (D), adductor longus (E), and soleus (F) muscles in AL and CR treated groups. *$p<0.05$, CR versus AL. Data are means ± SEM. n=5-8 muscles per diet group

CHAPTER VI

DISCUSSION

Focus of this Discussion

This chapter of the dissertation will first summarize the major new results of Studies 1 to 3. A concise summary of the results from the three studies in this dissertation is provided in Table 6.1 (page 124). The chapter will also provide a brief discussion that integrates the insights gained by considering together the novel results from each study to extend our current understanding of CR-related increase on insulin-stimulated glucose uptake by sequentially addressing the novel insights from this dissertation with regard to CR effects on: insulin signaling proximal to Akt, activation of Akt, phosphorylation of Akt substrates, and glucose uptake. This discussion will also identify some of the key issues for future research that these studies brought to the forefront. The final section of this discussion will focus on how the contributions from the research included in the dissertation have provided a unique and important new perspective to understanding the influence of CR on insulin signaling and glucose uptake in skeletal muscle.

Summary of Key Findings

Results from this dissertation provide novel insights into fully understanding the mechanisms that account for enhanced insulin-stimulated glucose transport with calorie restriction (CR). Study 1 provided the first data on the effects of CR in old age on insulin signaling and glucose uptake in skeletal muscle with a physiologic insulin dose. Results from Study 2 evaluated the possibility that CR results in a transiently greater insulin-mediated activation in skeletal muscle of key insulin signaling that are proximal to Akt. Study 3 extended the observations based on evaluation of four predominantly fast-twitch and two predominantly slow-twitch skeletal muscles in vivo by probing CR effects on insulin-stimulated glucose uptake and insulin signaling in an in vivo model. A bulleted summary of the key findings is provided below.

Study 1: Calorie Restriction Enhances Insulin-stimulated Glucose Uptake and Akt Phosphorylation in Both Fast-twitch and Slow-twitch Skeletal Muscle of 24 Month-old Rats

- In male 24 mo-old Fisher 344 Brown Norway rats, CR (6 mo duration) glucose uptake was enhanced for both the insulin-stimulated soleus (primarily type I fibers) and epitrochlearis (primarily type II fibers).
- GLUT4 abundance was unchanged by CR in the soleus and epitrochlearis.
- Insulin receptor phosphorylation was not altered by diet in either soleus or epitrochlearis either with or without insulin.
- Akt phosphorylation on Thr308 and Ser473 was enhanced in the insulin-stimulated soleus and epitrochlearis.

- AS160 phosphorylation at Thr642 and Ser588 was unchanged by diet in the soleus and epitrochlearis either with or without insulin.
- TBC1D1 phosphorylation at Thr596 was unchanged by diet in both soleus and epitrochlearis either with or without insulin.
- Filamin C phosphorylation at Ser2213 was greater for CR versus AL rats in the insulin-stimulated soleus and epitrochlearis.

Study 2: Comparison of Ad Libitum Fed and Calorie Restricted Rats for the Time Course of Insulin's Activation of Signaling Steps Required for Greater Glucose Transport by Isolated Skeletal Muscle

- In male 9 mo-old Fisher 344 Brown Norway rats, 2-DG uptake by the insulin-stimulated soleus was significantly increased for the CR versus AL group.
- There was no significant difference between CR and AL rats for soleus phosphorylation of the IR on Tyr1162/1163 at any time point, either with or without insulin.
- Soleus tyrosine phosphorylation of IRS-1 was not significantly different between CR and AL rats regardless of insulin dose or time point.
- IRS-1 associated PI3K in the soleus muscles was not significantly different for the CR versus AL group at any time point, either with or without insulin.
- There was no significant effect of diet for either Akt Thr308 or Ser473 phosphorylation in the soleus at any time point under basal conditions.

However, phosphorylation of Akt on both Thr308 and Ser473 was significantly increased for the CR versus AL group in muscles that were incubated with insulin for 50 minutes, but not for muscles incubated with insulin for 5 or 15 minutes.

Study 3: In Vivo Assessment of the Effects of Calorie Restriction on Insulin-stimulated Glucose Uptake and the Insulin Signaling Pathway in Multiple Skeletal Muscles

- Whole body insulin sensitivity measured by glucose infusion rate during a euglycemic-hyperinsulinemic clamp was greater for CR versus AL rats that were studied with similar plasma insulin concentrations.
- There was increased 2-DG uptake of the epitrochlearis, gastrocnemius, and tibialis anterior for insulin-stimulated CR versus AL rats and no significant difference between diet groups for 2-DG uptake of the plantaris, adductor longus, and soleus.
- Phosphorylation of IR was not different between insulin-stimulated AL and CR rats for any of the muscles studied.
- The most consistent effect of CR compared to AL was a robust increase in insulin-stimulated Akt phosphorylation found at both the Thr308 and Ser473 sites in the epitrochlearis, gastrocnemius, tibilais anterior, and soleus muscles.

- Akt Ser473 phosphorylation was increased for CR versus AL rats in the plantaris muscle, and there was a strong statistically non-significant trend (P=0.07) for an increase at Akt Thr308.
- Only the adductor longus did not significantly differ between AL and CR for Akt phosphorylation at either the Thr308 or the Ser473 site.
- AS160 (Thr642 and Ser588) phosphorylation was greater for CR versus AL in the insulin-stimulated epitrochlearis and tibialis anterior.
- AS160 phosphorylation at Thr642, but not at S588, was greater for CR versus AL rats in the insulin-stimulated soleus muscle, and the plantaris had a statistically non-significant trend to increase at Ser588 (P=0.06) without a diet effect at Thr642.
- There was no evidence for an increased AS160 phosphorylation in the gastrocnemius or adductor longus.
- Filamin C phosphorylation on Ser2213 was greater for CR versus AL rats only in the insulin-stimulated plantaris muscle.

Insights from Integrating the Results of Studies 1 to 3

The studies in this dissertation investigated the mechanisms for calorie restriction (CR) effects on insulin-simulated glucose uptake in skeletal muscle using rats of different ages (9 or 24 mo-old) and using different experimental models for measuring glucose uptake (ex vivo or in vivo). Together these studies offer new insights into possible mechanisms for CR-related benefits on insulin sensitivity in muscle. This discussion will sequentially address the specific results for the insulin signaling pathway and then comment on the functional outcome of glucose uptake, with ideas for future research

included for each topic. Table 6.1 summarizes the experimental design and results for the study by Sharma et al. and the studies from this dissertation. The following section integrating results from Studies 1 to 3 will periodically refer to table 6.1.

Table 6.1 Summary of experimental design and results for study by Sharma et al. (24) and Studies 1 to 3 from dissertation.

	Sharma et al. (24)	**Study 1**	**Study 2**	**Study 3**
Model Used for Insulin Exposure	Ex Vivo	Ex Vivo	Ex Vivo	In Vivo
Age of Rats	9 mo-old	24 mo-old	9 mo-old	9 mo-old
Muscles Studied	E & S	E & S	S	E, S, G ,T, P & A
Insulin-stimulated pY-IR	↔ E ↔ S	↔ E ↔ S	↔ S	↔ (E, S, G ,T, P & A)
Insulin-stimulated pY-IRS1	not measured	not measured	↔ S	not measured
Insulin-stimulated IRS1-PI3K	↔ E ↔ S	not measured	↔ S	not measured
Insulin-stimulated pAkt	↑ E ↑ S	↑ E ↑ S	↑ S	↑ (E, S, G, T & P); ↔ A
Insulin-stimulated pAS160	↑ E ↔ S	↔ E ↔ S	not measured	↑ (E, S, T & P); ↔ (G & A)
Insulin-stimulated 2DG Uptake	↑ E ↑ S	↑ E ↑ S	↑ S	↑ (E, G & T); ↔ (S, P & A)

KEY: E = Epitrochlearis; S=Soleus; G=Gastrocnemius; T=Tibialis Anterior; P=Plantaris; A=Adductor Longus; ↑ = CR significantly greater than AL for corresponding measurement; ↔ = no significant difference for CR versus AL for corresponding measurement.

Table 6.2 Results of 2-way ANOVA showing main effects of insulin and diet and post-hoc analysis for Study 1.

	Main Effect of Insulin	**Main Effect of Diet**	**Post-hoc AL vs. CR with no insulin**	**Post-hoc AL vs. CR with 1.2 nM insulin**
Insulin-stimulated pY-IR	**EPI:** P < 0.05 **SOL:**P < 0.001	**EPI:** P = 0.384 **SOL:**P = 0.311	**EPI:** P = 1.00 **SOL:**P = 1.00	**EPI:** P = 1.0 **SOL:**P= 1.00
Insulin-stimulated pAkt T308	**EPI:** P < 0.001 **SOL:** P < 0.001	**EPI:** P < 0.001 **SOL:** P < 0.001	**EPI:** P = 1.00 **SOL:** P = 1.00	**EPI:** P< 0.001 **SOL:** P< 0.001
Insulin-stimulated pAkt S473	**EPI:** P < 0.001 **SOL:** P < 0.001	**EPI:** P < 0.001 **SOL:** P < 0.001	**EPI:** P = 1.00 **SOL:** P = 1.0	**EPI:** P< 0.001 **SOL:** P< 0.001
Insulin-stimulated pAS160 T642	**EPI:** P < 0.001 **SOL:** P < 0.001	**EPI:** P =0.198 **SOL:** P = 0.388	**EPI:** P = 1.00 **SOL:**P = 0.699	**EPI:** P = 0.341 **SOL:**P = 1.00
Insulin-stimulated pAS160 S588	**EPI:** P < 0.01 **SOL:** P < 0.001	**EPI:** P = 0.310 **SOL:** P = 0.051	**EPI:** P = 1.00 **SOL:**P = 1.00	**EPI:** P = 0.850 **SOL:**P = 0.203
Insulin-stimulated pTBC1D1 T596	**EPI:** P = 0.108 **SOL:** P = 0.194	**EPI:** P = 0.301 **SOL:** P = 0.979	**EPI:** N/A **SOL:**N/A	**EPI:** N/A **SOL:**N/A
Insulin-stimulated pFilaminC S2213	**EPI:** P < 0.005 **SOL:** P < 0.001	**EPI:** P < 0.05 **SOL:** P < 0.001	**EPI:** P = 1.00 **SOL:**P = 1.00	**EPI:** P = 0.055 **SOL:**P < 0.001
Insulin-stimulated 2DG Uptake	**EPI:** P < 0.005 **SOL:** P < 0.001	**EPI:** P < 0.01 **SOL:** P < 0.01	**EPI:** P = 1.00 **SOL:**P = 1.00	**EPI:** P < 0.05 **SOL:**P < 0.05

KEY: Epi = Epitrochlearis; Sol=Soleus; N/A= Not applicable; Post-hoc AL vs. CR refers to the results of the post-hoc Bonferroni test for each incubation for insulin exposure during ex vivo incubation (with no insulin or with 1.2 nM insulin). P value <0.05 was considered statistically significant.

CR Effects on Insulin Signaling Proximal to Akt

Insulin receptor phosphorylation did not increase with CR versus AL regardless of age (9 or 24 months) or model (ex vivo or in vivo). Muscles from CR versus AL rats did not differ significantly for $IR^{Tyr1162/1163}$ phosphorylation, IRS-1^{Tyr} phosphorylation, or IRS-1-associated PI3K activity, when tested at multiple time points (5, 15 and 50 minutes). These results suggest that the CR-related amplification of post-receptor signaling and glucose uptake in muscle stimulated by a physiologic insulin dose do not appear to be attributable to greater activation of insulin signaling proximal to Akt. Supporting the results of Study 2 of this dissertation, previously published research indicated a lack of a CR effect on IRS1-, IRS2, or phosphotyrosine PI3K was reported with 3 nM insulin in the epitrochlearis (25). Sharma et al. (24) observed no CR effect on PI3K activity together with no evidence for a CR effect on atypical PKC activity. The lack of any change in atypical PKC activity with CR, a step that is dependent on the proximal signaling events that also regulate Akt (including insulin receptor and IRS-1 tyrosine phosphorylation and IRS-1-PI3K activity) is consistent with a lack of a general increase in proximal signaling with CR, and it supports the idea of CR does not uniformly enhance all signaling events that are modulated by insulin via activation of PI3K activity.

Nonetheless, it remains possible that the lack of a statistically detectable CR effect on proximal signaling concomitant with a consistently significant CR effect on Akt could potentially be related to differences in the technical precision of the methods used to quantify the different insulin signaling steps. The dynamic range was smaller and the variability was greater for proximal signaling markers versus Akt signaling. It is possible that the lack of a statistically detectable CR effect of proximal signaling steps in this

study was due to insufficient sample size and statistical power. Only 3 time points were assessed and a difference may have been missed at an intermediate time point that was not studied. However, it is important to note that significant insulin effects were detected for proximal signaling in Studies 1 (Table 6.2 includes the P-values for insulin main effects based on 2-Way ANOVA) and 2 (Figures 4.2 to 4.5) of this dissertation, indicating that there was sufficient technical precision and statistical power to discern the effects of a physiologic insulin dose on each of the proximal insulin signaling steps measured for ex vivo epitrochlearis and soleus muscles. In addition, a recently published study from our group also found significant effects of insulin infusion compared to basal conditions (without insulin infusion) on insulin receptor tyrosine phosphorylation in multiple muscles studied under hyperinsulinemic clamp conditions identical to those used in Study 3 (115). However, it remains possible that a small and/or variable CR-related increase occurred for one or more proximal insulin signaling step, but was missed because it was below the limit of detection of the technical approaches that were used.

CR Effects on Activation of Akt

Akt phosphorylation was greater for CR versus AL for each age (9 versus 24 months) and for each model (ex vivo and in vivo). Thus, in striking contrast to the lack of detectable CR effects on insulin signaling at steps proximal to Akt, there was a substantial and significant CR effect on Akt phosphorylation in all of the studies of the dissertation. The simplest interpretation of the results of this dissertation is that the increase in Akt phosphorylation does not appear to be attributable to CR effects on key upstream signaling steps. What are some possible mechanisms whereby CR may be

affecting insulin sensitivity by distinctly acting on the level of Akt signaling without the need for enhanced signaling at proximal steps?

One possible explanation for increased Akt phosphorylation for CR versus AL could be due to a CR effect that results in attenuated action of the relevant protein phosphatases that are responsible for dephosphorylation of Akt. Several studies have demonstrated that protein phosphatase 2A (PP2A) can regulate kinase activity such as Akt phosphorylation (143-146). However, CR was reported to have no effects on Akt associated PP2A in the epitrochlearis and soleus muscles from 9 mo-old rats (24). PP2A has also been shown to be regulated by tyrosine 307 phosphorylation (147-149) and leucine 309 methylation (150-153), but the effects of CR on these post-translational modifications is unknown. PP2A is one of several phosphatases that regulate protein serine/threonine phosphorylation in skeletal muscle (154-158), and it is possible that other phosphatases (PP1, PP2B, PP2C) may be altered with CR.

A second possibility is that there are other post-translational modifications (e.g. acetylation) of Akt are influenced by CR and modulate Akt activation. The Sirt1 deacetylase has been reported to promote localization and activation of Akt (159, 160) and represents a mechanism that may play a role in the increase in Akt activation with CR. CR was reported to increase Sirt1 deacetylase activity in skeletal muscle of mice (117), but CR effects on Akt acetylation are currently unknown.

A third possibility is that regulatory proteins that are known to bind to and modulate Akt phosphorylation may be altered by CR. Heat-shock protein 90 (HSP90) has been reported to oppose dephosphorylation of Akt by protein phosphatases (161). Sharma et al (24) studied the effect of CR on HSP90 and found that CR increased the

association of HSP90 and Akt with insulin stimulation in the epitrochlearis muscle. However, there was no evidence for increase Akt and HSP90 association in the soleus muscle suggesting that different mechanisms may account for greater Akt phosphorylation in the soleus of CR rats. At least in the epitrochlearis, the CR mediated increase in HSP90-Akt binding may be significant for the CR-enhancement of Akt phosphorylation. Specific HSP90 inhibitors (162, 163) could be used to determine if HSP90 is playing a significant role in the CR effect on Akt phosphorylation that is routinely seen in skeletal muscle. Several other proteins have been reported to favor either greater Akt phosphorylation [e.g. PHLDB1(118), ClipR-59 (119)] or less Akt phosphorylation [e.g CTMP (120), TRB3 (121)]. Co-immunoprecipation of Akt and these various Akt-binding proteins in multiple muscles from CR compared to AL rats would provide clues on how CR acts to enhance Akt phosphorylation. It seems possible that CR actions on Akt–binding proteins may also influence the localization of Akt and influence the spatial organization to regulate Akt's co-localization with specific Akt substrates. To further probe this idea, specific inhibitors or genetic approaches to manipulate the expression of these candidate proteins would be useful. For example, siRNA that is specific to each candidate protein could be transfected into the skeletal muscle of CR and AL rats to determine if CR enhanced Akt association with Akt-binding proteins are important for the CR-mediated increase in Akt phosphorylation. Similarly, altering binding of Akt to Akt binding proteins by mutations on key binding amino acids would be an additional method to determine the significance of their association. Alternatively, overexpression experiments could also shed light on the importance of these Akt-binding proteins. Electrotransfection experiments could be used to up-regulate

plasmid DNA of interest using in vivo gene delivery to skeletal muscle. These approaches would provide important information on the regulation of Akt phosphorylation by Akt binding proteins.

CR Effects on Phosphorylation of Akt Substrates

Akt, the insulin signaling protein that has been found to be most consistently altered in response to CR, is a kinase that phosphorylates downstream substrates that are necessary for the CR-mediated enhancement of insulin stimulated glucose uptake in skeletal muscle. AS160 is the most studied Akt substrate that has been implicated in regulating glucose transport. AS160 was evaluated in the soleus for Studies 1 and 3, but was not studied for the soleus ex vivo in 9 mo-old rats. However, this information is available from an earlier study by Sharma et al. (24) (see Table 6.1) that was a precursor and template for the design of the 3 studies in this dissertation (with regard to the strain and sex of the rats, the specific CR protocol used, the insulin dose evaluated, and many of the key measurements that were made). Therefore, if we consider the results of Studies 1 and 3 together with the results of Sharma et al. (24) for AS160, it is apparent that CR does not increase AS160 phosphorylation in the soleus ex vivo in either 9 or 24 mo-old rats, even though CR is characterized by greater insulin-stimulated glucose uptake in the soleus at both ages. However, data from Study 3 indicate that CR does result in greater insulin-stimulated AS160 phosphorylation in the soleus when measured in vivo in the absence of a CR-related increase in insulin-stimulated glucose uptake. Taken together, the results for the soleus demonstrate that greater AS160 phosphorylation is not responsible for diet-related differences in glucose uptake by the soleus of CR versus AL rats.

AS160 phosphorylation in the ex vivo epitrochlearis was greater for CR versus AL in 9 mo-old rats, but there was no evidence of a CR-related increase in AS160 phosphorylation in the ex vivo epitrochlearis of 24 mo-old rats. It is unclear what links elevated Akt phosphorylation to enhanced glucose uptake in old rats, but the lack of greater AS160 phosphorylation in the epitrochlearis of old CR rats suggests that alternate Akt substrates may be enhanced and account for greater glucose uptake with CR. It would be worthwhile to probe CR effects on new Akt substrates to reveal potential mechanisms that may explain the CR improved glucose uptake in 24 mo-old rats. CDP138, an Akt substrate, was reported to regulate GLUT4 insertion into the plasma membrane (164) and may be relevant for the CR effect on insulin-stimulated glucose uptake in the epitrochlearis and other skeletal muscles.

This dissertation also evaluated the effect of CR on in vivo insulin induced activation of AS160 in the epitrochlearis. When the CR effects on AS160 phosphorylation were probed in vivo there was a significant increase in the epitrochlearis for CR versus AL in 9mo-old rats. Comparing the AS160 phosphorylation for ex vivo and in vivo, the data provides evidence that AS160 phosphorylation is responsive to the effects of CR in the epitrochlearis. Three additional predominantly fast-twitch muscles were studied in 9 mo-old CR and AL rats, and there was evidence for a CR-related increase in AS160 phosphorylation in the tibialis anterior and plantaris muscles but no effect of CR on AS160 phosphorylation in the gastrocnemius. The lack of a uniform effect of CR on AS160 phosphorylation in the 4 fast-twitch muscles that were studied in vivo is not attributable to differences in these muscles for CR effects on Akt

phosphorylation, because all 4 of the fast-twitch muscles responded to CR with greater Akt phosphorylation.

Filamin C is a protein that was recently discovered in research using cultured cells to be regulated by Akt-dependent phosphorylation in response to insulin (76). Prior to the completion of this dissertation, there was no published research on insulin's effects on the phosphorylation of filamin C in skeletal muscle tissue (i.e., only in cultured cells). This dissertation demonstrated phosphorylation of filamin C in insulin-stimulated muscles from 24 mo-old rats in Study 1 to be responsive to CR. Filamin C increased with CR in Study 1 in 24 mo-old rats in epitrochlearis and soleus muscles. However, in Study 3, filamin C only increased in 1 out of 6 muscles studies in vivo, and glucose uptake was not enhanced by CR for this muscle (the plantaris), indicating that greater filamin C phosphorylation does not account for CR effects on muscle glucose uptake in vivo. Although these results argue against greater phosphorylation of filamin C being essential for CR effects on in vivo glucose uptake by skeletal muscle, it remains possible that filamin C is important for insulin-stimulated glucose uptake in skeletal muscle. Therefore, it would be valuable to perform experiments with serine to alanine mutations on the Ser2213 site to test if the ability to phosphorylate this site is important for insulin-stimulated glucose uptake.

CR does not uniformly enhance phosphorylation of all Akt substrates and this may be due to localization in certain subcellular regions. It is possible that the inability to recognize a CR effect on particular signaling proteins in skeletal muscle may be the result of measuring the signaling proteins in whole muscle lysates. Subcellular localization of proteins is crucial for their functional capacity, and localization of insulin signaling

proteins to the plasma membrane is necessary for their ability to regulate the GLUT4 transporter and glucose transport (165). Therefore, measuring specialized pools of key signaling proteins may reveal CR effects on insulin-stimulated samples that previously were masked by measuring whole cell lysates. It is still not entirely clear how Akt substrates implicated in GLUT4 translocation function with CR.

CR Effects on Glucose Uptake

The effects of CR on insulin signaling were examined to probe the potential mechanism of the CR-enhancement of insulin-stimulated glucose uptake in skeletal muscle. Glucose uptake is a rate-limiting step in skeletal muscle glucose metabolism and represents a key regulatory step improved by CR. Thus, insulin-stimulated glucose uptake was the most significant functional endpoint measurement that was studied in this dissertation.

Studies 1 and 3 included data for the predominantly fast-twitch epitrochlearis muscle, but the dissertation did not include results for ex vivo epitrochlearis muscles from 9 mo-old rats. However, this information is available from the earlier study by Sharma et al. (24). Therefore, the next section of the discussion will consider the results of Studies 1 and 3 together with those of Sharma et al. for the epitrochlearis. 2DG uptake increased with CR versus AL in the epitrochlearis regardless of age (9 versus 24 mo-old) or model (ex vivo or in vivo). Thus, there was a consistent CR effect on glucose uptake by the epitrochlearis in every model in this dissertation. It might be suspected that because the epitrochlearis is a predominantly fast-twitch muscle, that it may also be true that other predominantly fast-twitch muscles are also very responsive to the CR-mediate increase in insulin-stimulated glucose uptake. However, it would be unwise to

make this generalization based only on the results of a single muscle. The epitrochlearis along with three additional fast-twitch muscles (gastrocnemius, tibialis anterior and plantaris) were evaluated in Study 3. Although these muscles were not studied under ex vivo conditions because their large sizes makes them unsuitable for this procedure, it is possible to compare their results with the epitrochlearis in vivo data to evaluate the possibility of identifying fiber-type related patterns for the effects of CR. There was a significant CR effect on 3 of 4 predominantly fast-twitch muscle. The plantaris was the only predominantly fast-twitch muscle in which there was not a CR effect on insulin-stimulated glucose uptake. Because the MHC isoform makeup of the plantaris is similar to the other predominantly fast-twitch muscles in this study, fiber type composition does not seem to account for the lack of a CR-mediated increase in insulin-stimulated glucose uptake in the plantaris. It remains to be determined why CR had no effect on glucose uptake in the plantaris. The results of previous studies from a single muscle (e.g., the epitrochlearis) are often used to represent all predominantly fast-twitch muscles (3, 10, 21, 25, 85-87). The current results demonstrated that most, but not all (3 of 4) of the predominantly fast-twitch muscles studied were responsive to a calorie restriction-induced elevation in insulin-stimulated glucose uptake. Based on available evidence in this dissertation, there is not an obvious explanation to why there was not was not significant increase in insulin-stimulated glucose uptake for CR versus AL in the plantaris.

Enhanced insulin-stimulated glucose uptake with CR was observed in the epitrochlearis using both ex vivo and in vivo models. Sharma et al. reported a 61% increase in insulin-stimulated glucose uptake for CR versus AL. Similarly, in this study

there was a 71% increase in insulin-stimulated glucose uptake for CR versus AL in the epitrochlearis. Studying both models in the epitrochlearis allowed the comparison of factors intrinsic to the muscle (ex vivo) versus the combination of intrinsic and extrinsic factors (in vivo) that contributed to CR effects on the muscle. The fold increase ex vivo (~1.6-fold) was roughly similar to the fold increase in vivo (~1.7-fold), suggesting that the mechanism for the CR-enhancement on glucose uptake in the epitrochlearis is, at least in large part, intrinsic to the muscle because most of the CR effect on insulin-stimulated glucose appears to be present even in the absence of systemic factors. The results from the ex vivo condition also show that the effect of CR on insulin-stimulated glucose uptake in skeletal muscle persists in the presence of systemic factors extrinsic to the epitrochlearis. This shows that the benefit of CR by increasing insulin-stimulated glucose uptake is preserved in a more physiologically relevant environment and likely critical to the general benefits of CR on whole organisms.

Glucose uptake measured ex vivo in the soleus increased with CR versus AL for both 9 mo-old (Study 2) and 24 mo-old (Study 1) rats, but there was no significant CR-related increase when 2DG uptake was measured in vivo in the soleus. The difference for in vivo versus ex vivo glucose uptake by the soleus of 9 mo-old rats suggests that systemic factors may have eliminated the CR effects observed in isolated soleus muscles. It is possible that differences for the effect of CR on soleus glucose uptake measured in vivo versus ex vivo are related to differences in measurement methods used for the in vivo versus ex vivo experiments. Ex vivo glucose uptake measurement for Studies 1 and 2 and for the experiment by Sharma et al. (24) used the standard assay procedures that result in quantifying the accumulation of both 2-DG and 2-DG-6-P as described

previously (110, 141). In vivo glucose uptake was measured in Study 3 using the standard assay procedures that result in the accumulation of only 2-DG-6-P as described previously (126-128). Another possibility is that differences between the in vivo and ex vivo conditions (e.g., duration and concentration of insulin exposure; presence or absence of glucose when 2-DG uptake occurred) may have contributed to the differences with regard to CR effects on 2-DG uptake by the soleus measured in the two conditions. It is not possible to recapitulate the ex vivo incubations (no glucose during 2-DG accumulation) during in vivo glucose uptake measurement, but it is feasible to mimic many of the conditions of the in vivo experiment (2 hr insulin exposure in the presence of glucose, with radiolabelled 2-DG included during the final 30 min of insulin exposure) for the ex vivo glucose uptake measurement. It is also possible to measure both 2-DG and 2-DG-6-P accumulation as well as only 2-DG-6-P accumulation in the ex vivo muscles. Insulin-stimulated soleus and epitrochlearis muscles from AL and CR rats would be studied using the conditions that simulated the in vivo conditions during the clamp. After incubation, muscles would be divided and processed so that one portion could be used to quantify 2-DG-6-P alone and the other portion would be processed to quantify both 2DG-6-P and 2-DG. If necessary, follow-up studies could then be performed to probe the specific roles of insulin concentration, insulin exposure time, and inclusion of glucose during the 2-DG uptake assay to account for differing effects of CR on glucose uptake results.

Novel Insights and Perspectives from the Research in this Dissertation

The most unique and important feature of the research in this dissertation was probing the mechanisms of CR effects on insulin-stimulated glucose uptake by rat

skeletal muscles using multiple perspectives by exploring time-course effects, evaluating both ex vivo and in vivo models, and studying six different muscles. This strategy provided the most complete representation of CR on skeletal muscle glucose metabolism to date by combining all of the individual assessments in Study 1 to 3.

Overall, CR's most consistent effect on signaling was for Akt phosphorylation, regardless of age or experimental model (in vivo or ex vivo). From each perspective, Akt phosphorylation was enhanced with CR. Without exception, insulin-stimulated Akt phosphorylation increased without any detectable change in proximal insulin signaling when insulin-stimulated glucose uptake was increased in any skeletal muscle for CR versus AL. Taken together, these results provide evidence that strongly suggests that increased Akt phosphorylation is a key insulin signaling step that is important for the CR-related increased insulin-stimulated glucose uptake. It would be valuable for future experiments to focus on Akt signaling in order to establish causality of Akt phosphorylation on the CR-mediated increase in skeletal muscle insulin-stimulated glucose uptake.

Studies 1 and 3 evaluated CR effects on both Akt and two of its substrates, AS160 and filamin C in multiple muscles under both ex vivo and in vivo conditions. The more consistent effect of CR on Akt than on its Akt substrates reveals the complexity of their relationship with each other. It was clear that there was not a single uniform pattern for the activation of Akt substrates even though there was a consistent increase in Akt phosphorylation in almost every condition. This complex relationship between the CR effects on phosphorylation of Akt and Akt substrates may be related to CR effects on various regulatory factors, including altering the level of binding of proteins to Akt or to

the substrates, the actions of protein phosphatases, and/or the extent of co-localization of Akt and each of its substrate.

The similarity for CR-related effects on ex vivo and in vivo glucose uptake for the epitrochlearis, but not the soleus, suggests a muscle-specific difference in susceptibility to the influence of systemic factors. In vivo experimentation is advantageous because insulin-stimulated glucose uptake measurements are taken in a physiological context and includes the influence of usual-living insulin levels, blood flow, neural inputs, and blood chemokines that may influence CR's effects on skeletal muscle. It will be important that future experiments use in vivo models in combination with ex vivo experimentation. Conclusions made from ex vivo models provide a framework that should guide in vivo experimentation where effects can be quite distinct but more physiologically relevant.

A striking result of this dissertation was the demonstration that skeletal muscles with similar fiber type profiles can have different responses to CR for effects on in vivo insulin signaling and glucose uptake. The most interesting example of this heterogeneity was the plantaris muscle. The plantaris was the only predominantly fast-twitch muscle in which CR did not enhance in vivo insulin-stimulated glucose uptake. It is evident that there is not a simple fiber-type pattern that uniformly predicts a CR-related increase for insulin-stimulated glucose uptake. Akt and AS160 phosphorylation increased for CR versus AL in the plantaris, but there was no increase in insulin-stimulated glucose uptake. These observations support the conclusion that the CR-induced enhancement of insulin-stimulated activation of Akt may be necessary, but not sufficient for the CR-induced increase in insulin-stimulated glucose uptake. In vivo filamin C phosphorylation was increased for CR versus AL in the plantaris muscle which suggests that filamin C is

neither necessary nor sufficient for insulin-stimulated glucose uptake. The results of Study 1 indicated that filamin C may be important for the CR related increase in insulin-stimulated glucose uptake in ex vivo muscles from 24 mo-old rats. However, filamin C appears to be less important in 9 mo-old rats (versus 24 mo-old rats) and in vivo (versus ex vivo) for the CR related increase in insulin-stimulated glucose uptake.

The novel results of the research in this dissertation revealed that there are similarities and differences among different skeletal muscles of similar fiber type profiles, suggesting a complex interplay between each muscle's intrinsic properties and the extrinsic systemic factors, with the interaction between the intrinsic and extrinsic features accounting for the CR effects on each skeletal muscle. The effects of CR on insulin-stimulated glucose uptake in skeletal muscle are not simply attributable to the fiber type composition as might have been predicted based on earlier studies. The results of Study 3 confirm that greater whole body insulin sensitivity, at least in part, is attributable to substantial CR effects on in vivo insulin-stimulated glucose uptake by skeletal muscle. However, fully understanding the effects of CR on whole body insulin sensitivity will require additional research to elucidate the specific mechanisms that modulate the metabolic functions of multiple diverse skeletal muscles. Ultimately, the influence of CR across of the muscles and other tissues can be summed to create a mosaic that accounts for how CR leads to greater whole body insulin sensitivity.

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