Published in final edited form as:
Mech Ageing Dev. 2013 January ; 134(1-2): 60–63. doi:10.1016/j.mad.2012.12.002.

Greater filamin C, GSK3α, and GSK3β serine phosphorylation in insulin-stimulated isolated skeletal muscles of calorie restricted 24 month-old rats

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Abstract

Moderate calorie restriction (CR) can improve insulin-stimulated Akt phosphorylation and glucose uptake in muscles from 24 month-old rats, but the specific Akt substrates linking CR-effects on Akt to glucose uptake and other cellular processes are uncertain. We probed CR's influence on site-specific phosphorylation of five Akt substrates (AS160^{Ser588}, TBC1D1^{Thr596}, FLNc^{Ser2213}, GSK3α^{Ser21}, and GSK3β^{Ser9}) in predominantly fast-twitch (epitrochlearis) and predominantly slow-twitch (soleus) muscles. We observed no CR-effect on phosphorylation of AS160^{Ser588} or TBC1D1^{Thr596}, but there was a CR-induced increase in insulin-stimulated FLNc^{Ser2213}, GSK3α^{Ser21}, and GSK3β^{Ser9} phosphorylation for both muscles. These results indicate that CR does not uniformly affect insulin-mediated phosphorylation of Akt substrates in fast- or slow-twitch muscles from 24 month-old rats.

Moderate calorie restriction (CR; ~60% of ad libitum, AL, consumption) has multiple, diverse biological consequences (Omodei and Fontana, 2011), including improved insulin sensitivity which is secondary in large part to elevated insulin-stimulated glucose uptake in skeletal muscle (Cartee, 2008; Sharma et al., 2011). We recently assessed mechanisms for CR-induced improvement in insulin sensitivity of old rats by evaluating muscle insulin signaling and glucose uptake of 24 month-old AL and CR rats (Sequea et al., 2012). Consistent with results for 9 month-old rats (Sharma et al., 2011), CR induced greater insulin-stimulated Akt phosphorylation and glucose uptake in both predominantly fast-twitch (epitrochlearis) and predominantly slow-twitch (soleus) muscles of 24 month-old rats. Intriguingly, in contrast to 9 month-old rats (Sharma et al., 2011), there was no effect of diet in either muscle on Akt substrate of 160kDa (AS160) Thr642 phosphorylation, a downstream substrate of Akt, that is important for insulin-stimulated glucose uptake (Sano et al., 2003), suggesting another mechanism for CR's effect on insulin sensitivity in old rats.

Because greater insulin-mediated Akt activation in muscle is a reliable CR-effect (McCurdy and Cartee, 2005; McCurdy et al., 2003; Sequea et al., 2012; Sharma et al., 2011; Sharma et

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al., 2012a), it would be valuable to identify the specific Akt substrate(s) linking CR-effects on Akt to elevated glucose uptake. Akt's phosphorylation of AS160 on a second phosphosite (Ser588) is also important for insulin-mediated glucose uptake (Sano et al., 2003), and TBC1D1, a paralog of AS160, is another insulin-regulated Akt substrate that may modulate glucose uptake (Cartee and Funai, 2009; Sakamoto and Holman, 2008; Szekeres et al., 2012; Taylor et al., 2008). We recently demonstrated greater filamin C (FLNc) phosphorylation on an Akt phosphosite (Ser2213) in insulin-stimulated epitrochlearis muscles from 9 month-old CR versus AL rats (Sharma et al., 2012a). These results were interesting because insulin signaling proteins and GLUT4 transporter translocation depend on actin filaments (Fujita et al., 2012), and FLNc is an actin-binding protein and Akt substrate (Murray et al., 2004). Additionally, glycogen synthase kinase-3 isoforms (GSK3α and GSK3β) are Akt substrates that modulate numerous cellular processes including protein synthesis and glycogen metabolism (Henriksen and Dokken, 2006).

The goal of the current study was to extend the mechanistic insights from our recent research (Sequea et al., 2012; Sharma et al., 2011; Sharma et al., 2012a); by probing the influence of CR on site-specific phosphorylation of five Akt substrates (AS160^{Ser588}, TBC1D1^{Thr596}, FLNc^{Ser2213}, GSK3α^{Ser21}, and GSK3β^{Ser9}). We hypothesized that CR would enhance insulin-stimulated phosphorylation on one or more of these Akt substrates in epitrochlearis and soleus muscles from old rats.

Animal care procedures were approved by the University of Michigan Committee on Use and Care of Animals. CR (~60% of AL intake, beginning at 14 weeks of age; n=11) and AL (n=11) male Fischer 344 × Brown Norway, F1 generation (FBN) rats were obtained at 23 months-old from the National Institute of Aging Calorie Restriction Colony. When rats were 24 months-old, epitrochlearis and soleus were dissected out, and muscle strips were incubated in physiological media containing either 0 nM (basal) or 1.2 nM insulin for 50 min as previously described (Sequea et al., 2012).

Antibodies used were: anti-phospho AS160 Ser588 (pAS160^{Ser588}; #3028P2; B-Bridge International; Mountain View, CA); anti-phospho filamin C Ser2213 (pFLNc^{Ser2213}; #PB-131; Kinasource (Dundee, Scotland, UK); anti-phospho TBC1D1 Thr596 (pTBC1D1^{Thr596}; provided by Dr. Makoto Kanzaki, Tohuku University, Japan); anti-phosho-GSK3α/β Ser21/9 (pGSK3α^{Ser21}, pGSK3β^{Ser9}; #9331) and anti-rabbit IgG-horseradish peroxide conjugate (#7074) were from Cell Signaling Technology, Danvers, MA; and anti-sheep IgG-horseradish peroxidase conjugate (#12–342; EMD Millipore, Billerica, MA). Immunoblotting was performed as previously described (Sequea et al., 2012). 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.

In both the epitrochlearis and soleus, pAS160^{Ser588} (Fig. 1) and pTBC1D1^{Thr596} (Fig. 2) did not differ between AL and CR groups regardless of insulin concentration. In both the epitrochlearis and soleus, pFLNc^{Ser2213}, pGSK3α^{Ser21}, and pGSK3β^{Ser9} did not differ without insulin (Figures 3 and 4), but pFLNc^{Ser2213}, pGSK3α^{Ser21}, and pGSK3β^{Ser9} were significantly (P ≤ 0.05) greater in insulin-stimulated epitrochlearis and soleus for CR versus AL rats. We recently reported (Sequea et al., 2012) in isolated epitrochlearis and soleus muscles from 24 month-old male FBN rats, there was no change in total GLUT4 abundance or insulin-stimulated glucose uptake between AL and CR groups, but CR versus AL rats had significantly (P 0.05) greater insulin-stimulated glucose uptake in both the epitrochlearis and the soleus.

The novel results of this study, together with published results (Sequea et al., 2012; Sharma et al., 2011), revealed an apparent age-related difference for CR-effects on insulin-

stimulated AS160 phosphorylation. The lack of a CR effect on pAS160^{Ser588} for the epitrochlearis from 24 month-old rats differs from our prior demonstration of greater pAS160^{Ser588} for the epitrochlearis of 9 month-old rats (Sharma et al., 2011). Our previous research also indicated that CR resulted in greater values pAS160^{Thr642} in the epitrochlearis from 9 month-old rats (Sharma et al., 2011; Sharma et al., 2012a), but not from 24 month-old rats (Sequea et al., 2012). In the soleus, there were no significant CR-effects on either phosphosite for either 9 (Sharma et al., 2011) or 24 month-old rats. These results indicate that the CR-effects on AS160 phosphorylation are not uniform for all muscles or all ages.

In the absence of a CR-effect on AS160 phosphorylation in 24 month-old rats, one plausible scenario is that CR might influence phosphorylation of TBC1D1, an Akt substrate that can modulate glucose uptake. However, we found no evidence of enhanced pTBC1D1^{Thr596} in either epitrochlearis or soleus of 24 month-old rats. These results were consistent with our previous observation that CR did not alter TBC1D1 phosphorylation in either muscle from 9 month-old rats (Sharma et al., 2011) and suggest that CR effects in both muscles of old rats may rely on other Akt substrates.

The observation that insulin-stimulated pFLNc^{Ser2213} for both the epitrochlearis and soleus was greater for 24 month-old CR versus AL rats extended our earlier finding that pFLNc^{Ser2213} in isolated epitrochlearis was greater for CR versus AL in 9 month-old rats (Sharma et al., 2012a). Additionally, we previously found that incubating epitrochlearis muscles from 9 month-old CR rats with a dose of a highly selective Akt inhibitor that eliminated the CR-induced increase in Akt phosphorylation also eliminated the CR-induced increases in glucose uptake and pFLNc^{Ser2213} (Sharma et al., 2012a). Although insulin-regulated remodeling of actin filaments apparently has roles for both spatial localization of insulin signaling proteins and translocation of GLUT4 glucose transporter vesicles (Zaid et al., 2008), it remains to be determined if FLNc plays any role in the regulation of glucose uptake, and the functional significance of the observed CR-related increase in insulin-mediated pFLNc^{Ser2213} is unknown.

In 9-month old rats, we found CR resulted in a trend for increased insulin-stimulated phosphorylation of GSK3β^{Ser9} in the soleus (Sharma et al., 2012b), but there are apparently no previous reports of the effects of CR on insulin-stimulated GSK3α or GSK3β phosphorylation in muscle from older rats. The functional outcomes of the CR-induced increases in GSK3α and GSK3β phosphorylation in the insulin-stimulated epitrochlearis and soleus of 24 month-old rats in the current study are unknown. However, GSK3 activity is reduced by serine phosphorylation, which in turn would be expected to result in less phosphorylation of GSK3α and GSK3β substrates, and thereby predicted to influence multiple cellular processes, including protein synthesis and carbohydrate metabolism.

The most consistent CR-effect on insulin signaling in skeletal muscle is greater insulin-mediated activation of Akt, and we recently reported this CR-effect in epitrochlearis and soleus muscles from 24 month-old rats (Sequea et al., 2012). However, the current study demonstrated that CR can have diverse effects on the phosphorylation of Akt substrates in insulin-stimulated fast-twitch and slow-twitch muscles from 24 month-old rats. Future research should focus on: 1) identifying the mechanisms for the specificity of CR's effects on Akt substrates, and 2) determining the functional consequences of the CR-effects on insulin-stimulated phosphorylation of FLNc, GSK3α, and GSK3β, in skeletal muscle.

Acknowledgments

This work was supported by the National Institute on Aging grants AG-010026, AG-013283, and T32-AG000114.

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Highlights

- Calorie restriction (CR) can improve age-associated insulin resistance
- Insulin-stimulated Akt phosphorylation in muscle is a hallmark of CR
- No CR-effect on phosphorylation of 2 Akt substrates (AS160 or TBC1D1) in old rats
- CR caused greater insulin-stimulation of pFLNc^{Ser2213}, pGSK3α^{Ser21}, and pGSK3β^{Ser9}
- CR-effects on Akt substrate phosphorylation are not uniform in muscles of old rats

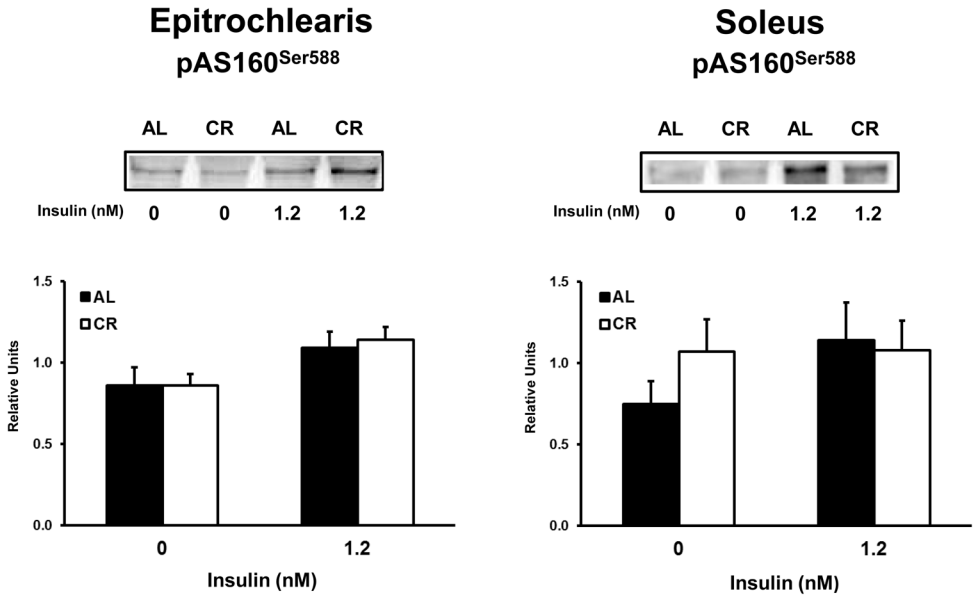

Figure 1.

AS160 Ser588 phosphorylation (pAS160^{Ser588}) in epitrochlearis (Left) or soleus (Right) muscles with 0 or 1.2 nM insulin. Filled bars = AL diet and open bars = CR diet. Values are means ± SEM, n = 6–11 per treatment group.

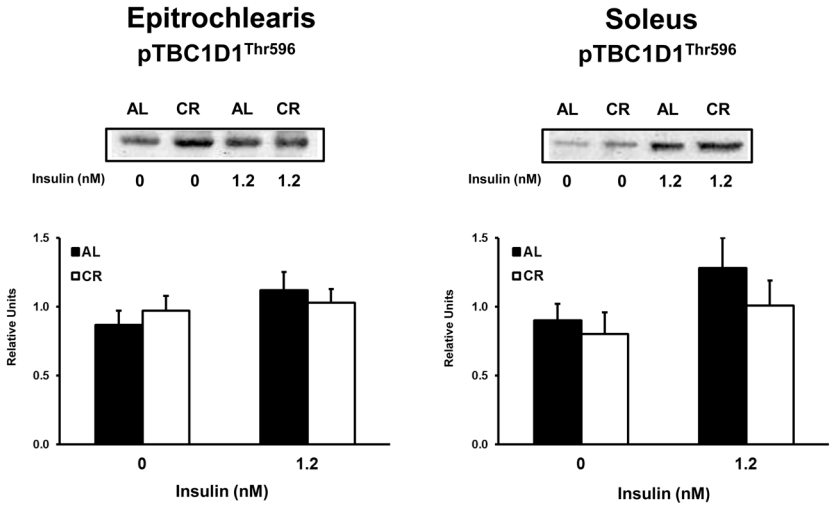

Figure 2.

TBC1D1 Thr596 phosphorylation (pTBC1D1^{Thr596}) in epitrochlearis (Left) or soleus (Right) muscles with 0 or 1.2 nM insulin. Filled bars = AL diet and open bars = CR diet. Values are means ± SEM, n = 6–8 per treatment group.

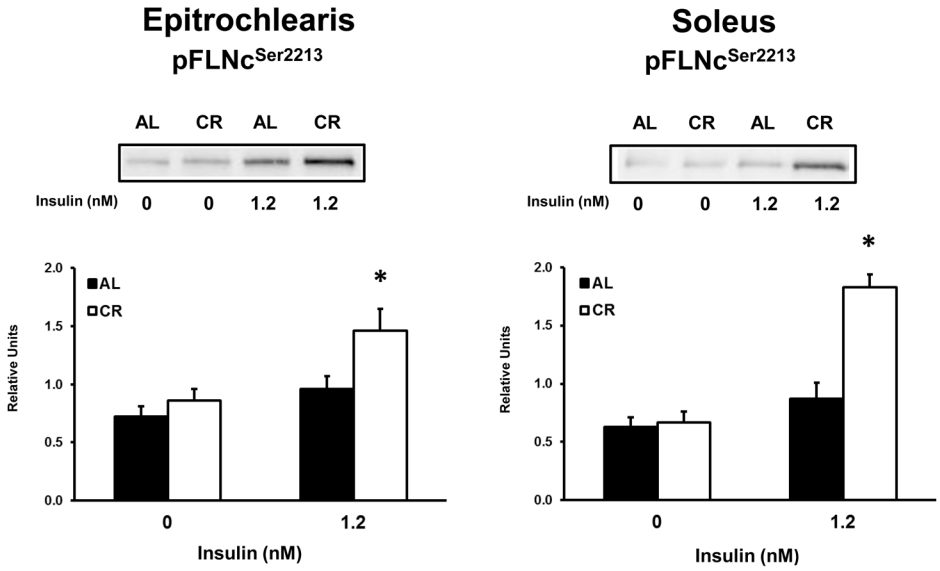

Figure 3.

Filamin C Ser2213 phosphorylation (pFLNc^{Ser2213}) in epitrochlearis (Left) or soleus (Right) muscles with 0 or 1.2 nM insulin. Filled bars = AL diet and open bars = CR diet. Values are means ± SE, n = 8 per treatment group. *P ≤ 0.05, CR versus AL in the same insulin treatment group.

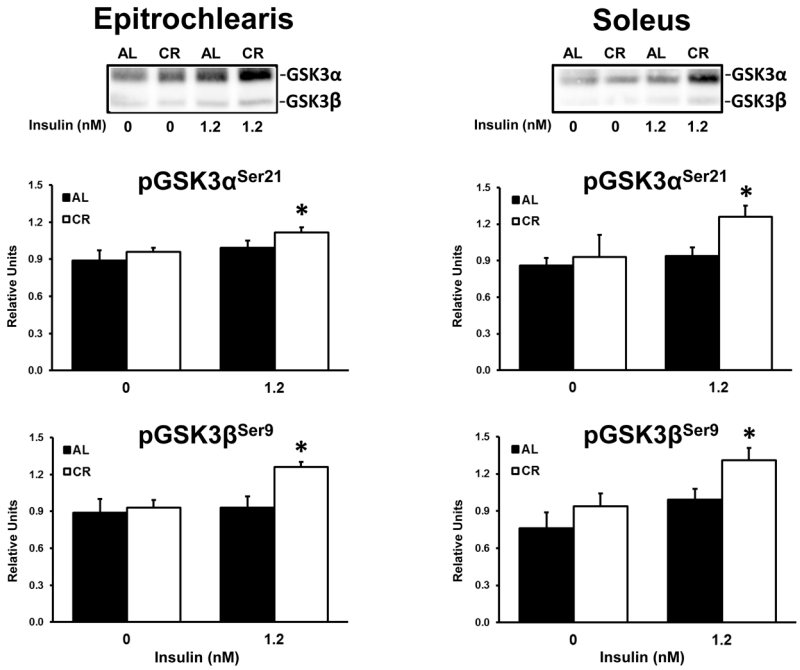

Figure 4.

GSK3α/β Ser21/9 phosphorylation (pGSK3α^{Ser21}, pGSK3β^{Ser9}) in epitrochlearis (Left) or soleus (Right) muscles with 0 or 1.2 nM insulin. Filled bars = AL diet and open bars = CR diet. Values are means ± SE, n = 8 per treatment group. *P ≤ 0.05, CR versus AL in the same insulin treatment group.