Improved insulin sensitivity with calorie restriction does not require reduced JNK1/2, p38, or ERK1/2 phosphorylation in skeletal muscle of 9-month-old rats

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Sharma N, Bhat AD, Kassa AD, Xiao Y, Arias EB, Cartee GD. Improved insulin sensitivity with calorie restriction does not require reduced JNK1/2, p38, or ERK1/2 phosphorylation in skeletal muscle of 9-month-old rats. Am J Physiol Endocrinol Metab 300: E966–E978, 2011.—Calorie restriction [CR; ~40% below ad libitum (AL) intake] improves the health of many species, including rats, by mechanisms that may be partly related to enhanced insulin sensitivity for glucose disposal by skeletal muscle. Excessive activation of several mitogen-activated protein kinases (MAPKs), including JNK1/2, p38, and ERK1/2 has been linked to insulin resistance. Although insulin can activate ERK1/2, this effect is not required for insulin-mediated glucose uptake. We hypothesized that skeletal muscle from male 9-mo-old Fischer 344/Brown Norway rats CR (35–40% beginning at 3 mo old) versus AL rats would have 1) attenuated activation of JNK1/2, p38, and ERK1/2 under basal conditions; and 2) no difference for insulin-induced ERK1/2 activation. In contrast to our hypothesis, there were significant CR-related increases in the phosphorylation of p38 (epitrochlearis, soleus, and gastrocnemius), JNK1 (epitrochlearis and soleus), and JNK2 (gastrocnemius). Consistent with our hypothesis, CR did not alter insulin-mediated ERK1/2 activation. The greater JNK1/2 and p38 phosphorylation with CR was not attributable to diet effects on muscle oxidative stress (assessed by protein carbonyls and 4-hydroxynonenal protein conjugates). In muscles from the same rats used for the present study, we previously reported a CR-related increase in insulin-mediated glucose uptake by the epitrochlearis and the soleus (Sharma N, Arias EB, Bhat AD, Sequea DA, Ho S, Croff KK, Sajan MP, Farese RV, Cartee GD. Am J Physiol Endocrinol Metab 300: E966–E978, 2011). The present results indicate that the improved insulin sensitivity with CR is not attributable to attenuated MAPK phosphorylation in skeletal muscle.

mitogen-activated protein kinases; insulin signaling; insulin resistance; dietary restriction; oxidative stress

A HALLMARK OF CALORIE RESTRICTION [CR; a 30–40% reduction in caloric consumption below ad libitum (AL) intake] is to improve skeletal muscle insulin sensitivity for glucose uptake (8, 9, 13, 16, 17, 41, 42, 54). The enhanced insulin sensitivity occurs even in nonobese individuals who undergo CR, and it is likely a key contributor to various positive health benefits found with CR (5, 22, 50, 51, 65). The mechanism for the improved insulin sensitivity appears to be related to enhanced activation of specific insulin-signaling proteins (8, 9, 13, 16, 17, 41, 42, 54).

The precise cellular processes that lead to greater insulin-stimulated activation of specific insulin-signaling proteins and glucose uptake remain to be identified. One potential scenario for the CR-induced improvement in skeletal muscle insulin sensitivity would be attenuated activation of proteins that restrain insulin-signaling and insulin-stimulated glucose uptake. Multiple members of a family of serine-threonine kinases called mitogen-activated protein kinases (MAPKs) can function as negative regulators of insulin sensitivity. Skeletal muscle highly expresses the JNK1 and JNK2 isoforms of c-Jun NH2-terminal kinase (6, 14), the ERK1 and ERK2 isoforms of extracellular signal-regulated kinase (62), and the p38α, p38β, and p38γ isoforms of p38 MAPK (43, 53). Excessive JNK activation has been frequently implicated in the development of insulin resistance (32, 34). There are also multiple lines of evidence linking greater stimulation of ERK1/2 to insulin resistance (11, 28, 35, 72, 73). Some studies have suggested that p38 can play a positive role in insulin-mediated glucose transport (24, 37), but other studies have provided evidence linking activation of p38 to insulin resistance (15, 18, 32).

Because previous studies have implicated excessive activation of JNK, ERK, and/or p38 in the development of insulin resistance (18, 28, 32, 34), it seems reasonable to speculate that CR might be accompanied by attenuated activation of these MAPKs in skeletal muscle. Therefore, our first aim was to determine whether skeletal muscles from adult (9-mo-old) CR (35–40% below AL for 6 mo duration) compared with AL rats differed with regard to their abundance and phosphorylation of ERK1, ERK2, p38, JNK1, and JNK2.

Oxidative stress has been linked to both the activation of MAPKs (48, 63) and the induction of insulin resistance (32). Furthermore, at least in older animals, CR has been reported to reduce markers of oxidative stress in skeletal muscle (27, 66). In this context, our second aim was to compare skeletal muscle from CR and AL rats with regard to markers of oxidative stress protein carbonyls and 4-hydroxynonenal (4-HNE protein conjugates).

Proteins other than MAPKs can also negatively modulate insulin signaling and insulin sensitivity. Very little is known about the influence of CR on many of these other proteins, including GSK-3 (19, 31, 39), mammalian target of rapamycin (mTOR) mTORC1 (57), and the IKKβ pathway (2, 52). Accordingly, our third aim was to determine whether skeletal muscle from CR versus AL rats differed with regard to phosphorylation and/or abundance of mTOR, GSK-3α, or GSK3-β or with regard to abundance of IkBα ( inversely-related to level of IKKβ activation).
Insulin has been reported to activate ERK in skeletal muscle from young rats (23, 25, 26, 36, 45). Although CR profoundly enhances the ability of insulin to activate Akt, CR does not uniformly enhance insulin-mediated activation of every protein that is regulated by insulin. For example, most (3, 4, 10, 54), but not all (59) studies have reported little or no CR-related effect on various markers of insulin receptor activation by physiologic insulin concentrations. In this context, our fourth aim was to determine the influence of CR on insulin-mediated ERK phosphorylation in skeletal muscle.

We hypothesized that skeletal muscles from CR versus AL rats would have attenuated activation of MAPKs, lower phosphorylation of GSK-3 and mTOR, reduced indices of oxidative stress, and greater abundance of IkBα. We further hypothesized that skeletal muscles from CR compared with AL rats would not differ with regard to the extent of insulin-induced ERK phosphorylation.

EXPERIMENTAL PROCEDURES

Materials

Reagents and apparatus for SDS-PAGE and immunoblotting were from Bio-Rad Laboratories (Richmond, CA). Anti-phospho-p44/42 (pERK1/2, Thr202/Tyr204; no. 9101), anti-phospho-p38 MAPK (Thr180/Tyr182; no. 9211), anti-phospho-mTOR (Ser2448; no. 9251), anti-phospho-SAPK/JNK (Thr183/Tyr185; no. 9251), anti-SAPK/JNK (no. 9252), anti-phospho-mTOR (Ser2448; no. 9271), anti-mTOR (no. 2972), anti-phospho-GSKα/β (Ser21/9; no. 9331), anti-GSK3α (no. 9338), anti-GSK3β (no. 9315), anti-IκBα (no. 9242), and anti-rabbit IgG-horseradish peroxidase conjugate (no. 7074) were purchased from Cell Signaling Technology (Danvers, MA). Anti-2,4-dinitrophenylhydrazone (DNP; no. 90451) was purchased from Thermo Scientific (Rockford, IL). All antibodies used were polyclonal, with the exceptions of anti-GSK3β and anti-DNP (monoclonal). Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Hanover Park, IL).

Animal Treatment

Procedures for animal care were approved by the University of Michigan Committee on Use and Care of Animals. Housing and feeding of these rats have been previously described (54). Male Fischer-344 × Brown Norway, F1 generation rats (F344 × BN) were obtained at 3 mo old from Harlan (Indianapolis, IN) and were individually housed and maintained on a 12:12-h light-dark cycle (dark at 1700 h). During an initial 2-wk acclimation period, rats had free access to food and water. Rats then had free access to food (NIH31 chow; Test Diet, Richmond, IN) and water for a second 2-wk acclimation period, where food consumption of all rats was measured daily between 1530 and 1630 h to determine baseline food intake. After the second acclimation period, rats were ranked by weight (lowest to highest) and alternately assigned to the ad libitum control group (AL) and the calorie restriction treatment group (CR) so that the initial mean weight and standard error were similar for both groups. The AL group (n = 13) received NIH31 chow ad libitum for the duration of the study, and the CR group (n = 12) received NIH31/NIA fortified chow (Test Diet). The CR group was then restricted to 60% of AL intake gradually over 3 wk (90%, 75%, 60%). Subsequently, the CR group received 60 to 65% of AL intake daily for ~6 mo (between 182 and 200 days). All CR rats were fed between 1530 and 1630 h each day and food intake of both groups was measured daily. CR intake was adjusted weekly to 60 to 65% of AL intake. All rats were weighed weekly at the same time of day (between 1530 and 1630 h).

Muscle Dissection and Incubation

On the morning of the experimental day when muscles were dissected, food was removed from the cages of all rats between 0700 and 0800 h. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg) between 1030 and 1130 h. When rats were deeply anesthetized, both soles and epimysial tissues were rapidly removed. One gastrocnemius muscle from each rat was also removed and immediately freeze-clamped using aluminum tongs cooled to the temperature of liquid nitrogen and stored at −80°C until analysis. By using a scalpel, each epimysial muscle was longitudinally transected into two strips of similar size and each soleus muscle was longitudinally transected into four strips of similar size. Muscle strips were incubated with 0 nM, 1.2 nM, or 30 nM insulin as previously described (54). During all incubation steps, muscles were placed in glass vials, warmed to 37°C in a heated water bath with shaking at 45 rpm, and continuously gassed with 95% O2-5% CO2. After incubation, the muscle strips were frozen as described above.

Muscle Lysate Preparation

Frozen muscles were weighed and transferred to prechilled glass tissue grinding tubes (Kontes, Vineland, NJ) and homogenized in ice-cold lysis buffer using a glass pestle attached to a motorized homogenizer (Cifam, Wiarton, ON). The lysis buffer contained T-PER (tissue protein extraction reagent; Thermo Scientific, Rockford, IL) augmented with 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM sodium vanadate, 1 mM β-glycerophosphate, 1 μM/μl leupeptin, and 1 mM PMSF. Homogenates were transferred to microfuge tubes, rotated for 1 h at 4°C, and then centrifuged at 15,000 g for 15 min at 4°C to remove insoluble material. Protein concentration was measured using the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL).

Immunoblotting

An equal amount of protein of each muscle sample was mixed with 6× Laemmli buffer, boiled for 5 min, and resolved with 10% SDS-PAGE before being transferred to nitrocellulose membranes. Membranes were then blocked in 5% milk or BSA in TBST (0.1% Tween-20) for 1 h at room temperature and transferred to 5% BSA-TBST with primary antibody at the appropriate dilution overnight at 4°C. Membranes were then washed three times for 5 min in TBST and incubated in anti-rabbit IgG horseradish peroxidase (1:20,000) for 1 h at room temperature. Blots were washed three times for 5 min in TBST, washed two times for 5 min in TBS (Tris-buffered saline, pH 7.5), and then subjected to enhanced chemiluminescence (cat. no. 34075, West Dura Extended Duration Substrate; Pierce, Rockford, IL) to visualize protein bands. Immunoreactive proteins were quantified by densitometry (AlphaEase FC; Alpha Innotech, San Leandro, CA). Two approaches were used to analyze the phosphorylated protein data. For the first approach, the unadjusted phosphorylated protein values for each sample were determined by densitometry after immunoblotting with the relevant phosphospecific antibody. For the second approach, the phospho-to-total ratio was calculated by dividing the phosphorylated protein value (determined using the first approach) by the corresponding value for total protein abundance obtained after immunoblotting the same sample using an antibody against the respective total protein (phospho-to-total ratio = phosphorylated protein value / total protein abundance value).

Protein Oxidation

Oxidized proteins (carbonyl groups) were assessed using the Oxyblot Protein Oxidation Detection Kit according to manufacturer’s instructions (cat. no. S7150; Millipore). Aliquots of each homogenized sample were treated as follows: one aliquot was derivatized with 2–4-dinitrophenyl-hydrazone (DNPH+), and a second aliquot served as a negative control (DNPH−) by substituting a derivatization-control solution for the DNPH solution. After the derivatization or negative control reaction, the samples were treated as previously described. The phospho-to-total ratio was then calculated by dividing the DNPH+ value by the DNPH− value.
were separated by SDS-PAGE and blotted with an antibody specific to the DNP moiety. We also evaluated 4-hydroxynonenal protein conjugates as a second indicator of oxidative stress (46). Proteins were separated by SDS-PAGE and probed with an anti-4-HNE antibody.

Statistical Analysis

For incubated soleus and epitrochlearis muscles, two-way ANOVA was used to determine significant main effects (diet and insulin treatment groups) and interactions (SigmaStat; SPSS, Chicago, IL). Bonferroni t-tests were used for post hoc analysis to identify the source of significant variance. For all of the other data, a Student's t-test was used for comparisons between AL and CR groups. Data are presented as means ± SE. P ≤ 0.05 was accepted as statistically significant.

RESULTS

Body Mass and Food Consumption

As expected, the final body mass for AL rats (377.4 ± 13.8 g) was significantly (P < 0.05) greater than for CR rats (211.1 ± 3.2 g). By design, the average daily food consumption of the AL group (18.1 ± 0.2 g) was greater (P < 0.05) than for the CR group (11.8 ± 0.3 g).

Protein Phosphorylation and Abundance

ERK1/2. ERK1 and ERK2 isoforms were independently quantified. In the epitrochlearis, there was a main effect of diet (CR > AL; P < 0.05) and a significant diet × insulin interaction (P < 0.05) on ERK1 phosphorylation (Fig. 1). There was also a nonsignificant trend (P = 0.068) for an insulin-related increase in pERK1. For ERK2 phosphorylation in the epitrochlearis, there was a main effect of insulin (CR > AL; P < 0.05) (Fig. 1). There was a main effect of diet (CR > AL; P < 0.05) for total ERK1 abundance (Table 1). When the values for phosphorylated ERK1 and ERK2 were expressed as a ratio to the values for total ERK1 and ERK2 abundance, respectively, the significant differences between the CR and AL groups for ERK1 and ERK2 phosphorylation were eliminated (Table 2).

Fig. 1. ERK1/2 Thr202/Tyr204 phosphorylation in epitrochlearis (left) and soleus muscles (right) with 0, 1.2, or 30 nM insulin. Phosphorylation of ERK1 for each muscle are shown (top), and phosphorylation of ERK2 are shown (bottom). Main effects of diet, insulin (Ins), and diet × insulin interactions from 2-way ANOVA are shown. AL, ad libitum fed group; CR, calorie-restricted group (−40% below AL group intake); NS, not significant. Data are means ± SE; n = 8 muscles per diet group and insulin concentration.
In the soleus, neither ERK1 nor ERK2 phosphorylation was significantly altered by insulin (Fig. 1). There was also no significant difference between AL and CR groups for ERK1 or ERK2 phosphorylation in the soleus. There was no significant effect of insulin or diet on total protein abundance of either ERK1 or ERK2 (Table 1).

In the gastrocnemius, neither ERK1 nor ERK2 phosphorylation was significantly altered by diet (see Fig. 4). Additionally, neither ERK1 nor ERK2 total abundance was altered by diet (Table 3).

JNK. JNK1 and JNK2 isoforms were independently quantified. In the epitrochlearis, there was a significant main effect of diet (CR > AL) on JNK1 phosphorylation (P < 0.05) (Fig. 2). There was no significant main effect of diet on JNK2 phosphorylation (Fig. 2). There was no significant main effect of insulin and no interaction between diet and insulin for either JNK1 or JNK2 phosphorylation (Fig. 2). There was no main effect of insulin or diet on total protein abundance of either JNK1 or JNK2 (Table 1).

In the gastrocnemius, JNK1 phosphorylation was not significantly altered by diet (Fig. 4). However, with JNK2, we observed a significant effect of diet (CR > AL; P < 0.05; Fig. 4). Neither JNK1 nor JNK2 total abundance was altered by diet (Table 3).

p38. We determined p38 phosphorylation with an antibody that recognized all four isoforms of p38 (α, β, γ, δ). In the epitrochlearis, there was a significant main effect of diet (CR > AL) on p38 phosphorylation (P < 0.05; Fig. 3). When expressed as a ratio of phosphorylated to total p38, there was not a significant diet effect in the epitrochlearis (Table 2). There was no significant effect of insulin or diet on total protein abundance of either JNK1 or JNK2 (Table 1). We determined p38 phosphorylation with an antibody that recognized all four isoforms of p38 (α, β, γ, δ). In the epitrochlearis, there was a significant main effect of diet (CR > AL) on JNK1 phosphorylation (P < 0.05) (Fig. 2). When expressed as a ratio of phosphorylated to total JNK1, there was no significant effect in the soleus. There was no main effect of diet on JNK2 phosphorylation (Fig. 2). There was no main effect of insulin and no interaction between diet and insulin for either JNK1 or JNK2 phosphorylation. There was no significant effect of insulin or diet on total protein abundance of either JNK1 or JNK2 (Table 1).

Table 1. Protein abundance in epitrochlearis and soleus

<table>
<thead>
<tr>
<th></th>
<th>0 nM AL</th>
<th>0.2 nM AL</th>
<th>0.4 nM AL</th>
<th>1.2 nM AL</th>
<th>3.0 nM AL</th>
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<tr>
<td>ERK1</td>
<td>0.717 ± 0.120</td>
<td>1.052 ± 0.103</td>
<td>0.951 ± 0.084</td>
<td>1.236 ± 0.119</td>
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<td>ERK2</td>
<td>0.929 ± 0.083</td>
<td>0.965 ± 0.065</td>
<td>1.051 ± 0.043</td>
<td>1.046 ± 0.098</td>
<td>1.039 ± 0.042</td>
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<td>JNK1</td>
<td>1.065 ± 0.109</td>
<td>0.939 ± 0.094</td>
<td>1.093 ± 0.091</td>
<td>1.105 ± 0.177</td>
<td>0.885 ± 0.048</td>
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<td>JNK2</td>
<td>1.073 ± 0.093</td>
<td>0.933 ± 0.073</td>
<td>1.141 ± 0.090</td>
<td>1.097 ± 0.085</td>
<td>1.004 ± 0.067</td>
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<tr>
<td>p38</td>
<td>1.293 ± 0.180</td>
<td>1.071 ± 0.152</td>
<td>1.179 ± 0.158</td>
<td>1.309 ± 0.180</td>
<td>1.084 ± 0.057</td>
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Table 2. Phosphorylated-to-total protein ratio in epitrochlearis and soleus

<table>
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<tr>
<th></th>
<th>0 nM AL</th>
<th>0.2 nM AL</th>
<th>0.4 nM AL</th>
<th>1.2 nM AL</th>
<th>3.0 nM AL</th>
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<tr>
<td>ERK1</td>
<td>1.153 ± 0.361</td>
<td>1.033 ± 0.137</td>
<td>0.931 ± 0.060</td>
<td>1.01 ± 0.111</td>
<td>1.081 ± 0.101</td>
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<td>ERK2</td>
<td>0.812 ± 0.133</td>
<td>0.865 ± 0.161</td>
<td>0.874 ± 0.069</td>
<td>0.950 ± 0.106</td>
<td>0.999 ± 0.065</td>
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<tr>
<td>JNK1*</td>
<td>0.923 ± 0.173</td>
<td>1.369 ± 0.214</td>
<td>0.841 ± 0.087</td>
<td>1.619 ± 0.278</td>
<td>1.108 ± 0.150</td>
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<td>JNK2</td>
<td>0.984 ± 0.129</td>
<td>1.367 ± 0.262</td>
<td>0.916 ± 0.146</td>
<td>1.425 ± 0.200</td>
<td>1.047 ± 0.165</td>
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<tr>
<td>p38*</td>
<td>1.364 ± 0.907</td>
<td>1.728 ± 0.742</td>
<td>0.847 ± 0.249</td>
<td>1.434 ± 0.215</td>
<td>0.791 ± 0.125</td>
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Values are means ± SE; n = 8 muscles per diet group and insulin concentration. *P < 0.05, main effect of diet and diet × insulin interaction.
with an antibody that recognized the α-, β-, and γ-isoforms. There was no significant effect of insulin or diet on total protein abundance of p38 (Table 1).

In the soleus, there was a main effect of diet (CR > AL) on p38 phosphorylation (P < 0.05; Fig. 3). There was no main effect of insulin and no interaction between diet and insulin.

There was no significant effect of insulin or diet on total protein abundance of p38 (Table 1). In the gastrocnemius, p38 phosphorylation was significantly different based on diet (CR > AL; P < 0.05; Fig. 4). Total abundance of p38 was unaltered by diet (Table 3).

mTOR. In the gastrocnemius, there was no significant effect of diet on mTOR phosphorylation (Fig. 5) or total protein abundance (Table 3).

GSK3α/β. In the gastrocnemius, there was no significant effect of diet on either GSK3α or GSK3β, for phosphorylation (Fig. 5) or total protein abundance (Table 3).

IκBα. In the gastrocnemius, there was no significant effect of diet on total IκBα total protein abundance (Table 3).

**Protein Oxidation**

We used DNP-specific immunoreactivity on carbonyl groups as a marker of protein oxidation in protein bands at four different molecular weights (25, 28, 43, and 67 kDa). We found no difference between the AL and CR groups for this

<table>
<thead>
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<th>AL</th>
<th>CR</th>
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<tbody>
<tr>
<td>ERK1</td>
<td>1.00 ± 0.19</td>
<td>1.10 ± 0.20</td>
</tr>
<tr>
<td>ERK2</td>
<td>1.00 ± 0.15</td>
<td>0.81 ± 0.20</td>
</tr>
<tr>
<td>JNK1</td>
<td>1.00 ± 0.05</td>
<td>0.92 ± 0.08</td>
</tr>
<tr>
<td>JNK2</td>
<td>1.00 ± 0.06</td>
<td>1.06 ± 0.14</td>
</tr>
<tr>
<td>p38</td>
<td>1.00 ± 0.08</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>mTOR</td>
<td>1.00 ± 0.11</td>
<td>0.92 ± 0.15</td>
</tr>
<tr>
<td>GSK3α</td>
<td>1.00 ± 0.08</td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>GSK3β</td>
<td>1.00 ± 0.04</td>
<td>0.85 ± 0.08</td>
</tr>
<tr>
<td>IκBα</td>
<td>1.00 ± 0.07</td>
<td>0.808 ± 0.09</td>
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</table>

Values are means ± SE; n = 8–12 muscles per diet group. mTOR, mammalian target of rapamycin.

Fig. 2. JNKThr183/Tyr185 phosphorylation in epitrochlearis (left) and soleus muscles (right) with 0, 1.2, or 30 nM insulin. Phosphorylation of JNK1 for each muscle is shown (top), and phosphorylation of JNK2 is shown (bottom). Main effects of diet, insulin, and diet × insulin interactions from 2-way ANOVA are shown. Additionally, there were significant increases (*P < 0.05; CR > AL) in JNK1 phosphorylation in the epitrochlearis with 1.2 nM insulin and in the soleus with 30 nM insulin as revealed by post hoc analysis. Data are means ± SE; n = 8 muscles per diet group and insulin concentration.
index of protein oxidation (Fig. 6). Additionally we used the 4-HNE antibody to detect protein conjugates that are indicative of oxidative stress. We quantified protein bands at four different molecular weights (38, 48, 63, and 92 kDa), and found no significant differences between the AL and CR groups (Fig. 7).

Insulin-Stimulated Glucose Uptake

As previously reported (54), glucose uptake rates by epitrochlearis and soleus muscle strips incubated with radiolabeled 2-deoxyglucose were measured for the same rats used in

Fig. 3. p38Thr180/Tyr182 phosphorylation in epitrochlearis (left) and soleus muscles (right) with 0, 1.2, or 30 nM insulin. Main effects of diet, insulin, and diet × insulin interactions from 2-way ANOVA are shown. Additionally, there was a significant increase (*P < 0.05; CR > AL) in p38 phosphorylation in the epitrochlearis with 1.2 nM insulin as revealed by post hoc analysis. Data are means ± SE; n = 8 muscles per diet group and insulin concentration.

Fig. 4. Phosphorylated ERK1, ERK2, JNK1, JNK2, and p38 in the gastrocnemius. *P < 0.05, CR vs. AL. Data are means ± SE; n = 8 rats per diet group.
the present study. The CR compared with AL rats had significantly greater ($P < 0.05$) glucose uptake ($\mu$mol $\times g^{-1} \times 20$ min$^{-1}$) in the epitrochlearis (1.2 nM insulin: $0.29 \pm 0.03$ vs. $0.18 \pm 0.01$; 30 nM insulin: $0.384 \pm 0.03$ vs. $0.27 \pm 0.01$) and the soleus (1.2 nM insulin: $0.55 \pm 0.04$ vs. $0.39 \pm 0.02$; 30 nM insulin: $0.73 \pm 0.04$ vs. $0.58 \pm 0.04$).

**DISCUSSION**

Contrary to our hypothesis, CR did not decrease the phosphorylation of any of the MAPks in any of the muscles that were studied. There was a CR-related increase in p38 phosphorylation in all three of the skeletal muscles that were studied (epitrochlearis, soleus, and gastrocnemius). The phosphorylation of JNK1 was also greater for CR versus AL rats in the epitrochlearis and soleus and tended to be greater for the gastrocnemius. JNK2 phosphorylation was increased for CR compared with AL rats in the gastrocnemius. The only significant diet effects on ERK were CR-related increases in both abundance and phosphorylation of ERK1 in the epitrochlearis. When the values for phosphorylated ERK1 were expressed relative to the values for total ERK1 abundance, the difference between the CR and AL groups for ERK1 phosphorylation was eliminated.

Two approaches were used to analyze the phosphorylated protein data: values were expressed either as a ratio or not as a ratio relative to the total abundance of the respective proteins. These two approaches provide complementary information. Expressing the data as a ratio is a mathematical adjustment for variable levels of total protein abundance. Significant differences between groups for the ratio values suggest that there may be a treatment effect on the regulatory mechanisms that alter the protein’s phosphorylation status (e.g., possible differences in the action of the kinases and/or phosphatases that modulate the relevant protein’s phosphorylation). Identifying differences in amounts of phosphorylated proteins without mathematical adjustment for total abundance is also informative because the unadjusted value is an index of the absolute amount of phosphorylated protein. Differences between groups for absolute levels of phosphorylated (i.e., activated) proteins would likely have functional consequences. Four of the phosphoprotein comparisons were significantly greater for CR vs. AL groups with either analytical approach (ratio or not): 1) the diet effect on pJNK1 in the epitrochlearis; 2) the diet effect on pJNK2 in the gastrocnemius; 3) the diet effect on p-p38 in the soleus; and 4) the diet effect on p-p38 in the gastrocnemius. Four of the comparisons that were statistically significant when not expressed as a ratio (CR > AL) were not statistically significant when expressed as a ratio: 1) the diet effect on pERK1 in the epitrochlearis; 2) the diet x insulin interaction on pERK1 in the epitrochlearis; 3) the diet effect on p-p38 in the epitrochlearis; and 4) the diet effect on pJNK1 in the soleus). One CR vs. AL comparison that was not statistically significant for values not expressed as ratio was significant when values were expressed as a ratio (the diet x insulin interaction for pJNK1 in the epitrochlearis). Ultimately, whether or not values were expressed as a ratio, none of the values for phosphorylated proteins were significantly greater for the AL vs. CR muscles. In summary, regardless of the analytical approach, these data provide no evidence that the CR-induced improvement in insulin sensitivity can be attributed to attenuated phosphorylation of ERK1, ERK2, JNK1, JNK2, p38, mTOR, GSK3α, or GSK3β.

The results were generally supportive of the hypothesis that isolated muscles from CR versus AL rats would not differ for insulin-mediated ERK phosphorylation. Apparently all of the previously published research that evaluated insulin effects on ERK phosphorylation in rat skeletal muscle studied much
younger animals ranging from 95 to 250 g (i.e., likely 1 to 2 mo old) (23, 25, 26, 36, 45, 67). These studies of rapidly growing rats found skeletal muscle ERK activation to be insulin responsive, whereas p38 and JNK phosphorylation were not consistently activated by insulin (12, 23, 25, 26, 36, 45, 67). There was a significant insulin-induced increase in ERK2 phosphorylation in the epitrochlearis and a significant diet×insulin interaction for ERK1 phosphorylation in the epitrochlearis. Insulin did not induce greater phosphorylation of ERK1 or ERK2 in the soleus. In an earlier study of very young rats that evaluated insulin’s effect on the activation of ERK in several muscles including the soleus, there was no evidence that the soleus was less responsive to insulin than either the red or white quadriceps (67). Several differences in experimental design might be responsible for the different results, e.g., the use of the perfused hindlimb of 200–250 g male Wistar rats (based on body wt, estimated to be 1.5 to 2 mo old) (67) versus isolated skeletal muscles in much older (9 mo old) male Fischer-344 × Brown Norway rats in the present study.

Using incubated muscle strips from the same rats used in the present study, we previously reported that glucose uptake rates by insulin-stimulated epitrochlearis and soleus muscles were greater for CR compared with AL rats (54). These data were consistent with the results of many other studies have demonstrated that insulin-mediated glucose uptake by skeletal muscle is also greater for CR compared with AL rats (8, 17, 42, 56). The results with isolated muscles are also consistent with the results of previous studies that have measured whole body insulin sensitivity in adult CR versus AL rats by various methods, including glucose infusion rate during a euglycemic-hyperinsulinemic clamp (23, 33).

Excessive JNK activation has been implicated in the development of insulin resistance (32, 34). Research using JNK1 null, JNK2 null, and wild-type mice found that the JNK1 null mice compared with the other genotypes were protected against high-fat diet-induced weight gain, hyperglycemia, and hyperinsulinemia (34). Some studies have suggested that p38 can play a positive role in insulin-mediated glucose transport (24, 37), but other studies have provided evidence linking activation of p38 to insulin resistance (15, 18, 32). It is notable that mice null for the MAP kinase phosphatase-1 compared with wild-type controls have elevated p38, JNK, and ERK.

Fig. 6. Detection of protein carbonyls in the gastrocnemius. Aliquots of each muscle are derivatized with 2–4-dinitrophenylhydrazine (DNPH) (+) or treated with a control solution [without DNPH (−)], and then proteins were subsequently separated by SDS-PAGE and blotted with a DNP-specific antibody (left). Bands at 25, 28, 43, and 67 kDa were quantified (right). MW, molecular weight. Data are means ± SE; n = 8 muscles per diet group.

Fig. 7. Detection of 4-hydroxynonenal protein conjugates in the gastrocnemius. Protein lysates were subsequently separated by SDS-PAGE and blotted with 4-hydroxynonenal antibody (left). Bands at 38, 48, 63, and 92 kDa were quantified (right). Data are means ± SE; n = 8 muscles per diet group.
activation in skeletal muscle and liver, but normal insulin-stimulated glucose disposal during a euglycemic-hyperinsulinemic clamp and normal activation of Akt in skeletal muscle and liver (68). The apparent paradox was reconciled by finding that the greater JNK activation was localized in the nucleus concomitant with reduced JNK activation in the cytosol. These results provide a precedent for the coexistence of normal insulin sensitivity together with elevated MAPK activation in skeletal muscle and other insulin target tissues.

What are possible mechanisms that underlie the CR-related increases in p38 and JNK phosphorylation? Given that p38 and JNK of incubated muscles were unresponsive to insulin and lower plasma insulin concentration is found for CR versus AL rats (40, 61), it is clear that the diet-related difference was not because of greater insulin-induced activation. There was no evidence for altered protein carbonyls or 4-HNE protein conjugates in skeletal muscle of CR rats in the present study, consistent with previous results for 8-mo-old CR rats (33). It seems unlikely that heat stress led to the diet-related effects on p38 and JNK activation because CR has been shown to lower the average body temperature of rats (20), and the incubated muscles from AL and CR rats were studied at the identical physiologic temperature. Corticosteroids have been shown to activate p38 and JNK (69), and plasma corticosterone has been reported to be higher for CR compared with AL rats (29, 30). The elevated p38 and JNK phosphorylation in cultured cells persisted for 2 h after removal of corticosterone (47). This sustained effect is relevant for interpreting the present study in which p38 and JNK phosphorylation was elevated both in muscle frozen immediately after removal (gastrocnemius) and in isolated muscles (epitrochlearis and soleus) incubated ex vivo without added corticosterone for 50 min were greater for the CR versus the AL rats. CR has been shown by others to increase plasma adiponectin concentration (55, 58, 74). This adipokine can induce the activation of p38 in cultured myocytes (38, 70, 71). It is uncertain whether corticosterone and adiponectin played any role in the diet-related differences in p38 and JNK activation.

In contrast to the CR-induced elevation in JNK phosphorylation in skeletal muscles of rats, Al-Regaiey et al. (1) found that CR caused a decrease in the phosphorylation of JNK1 and JNK2 of hindlimb muscle from 21-mo-old mice. An obvious difference between these studies was the age of animals being studied. JNK phosphorylation in skeletal muscle of AL animals has been reported to be increased in old compared with young adult rats (44) and mice (7), and in both of these studies, an age-related increase in oxidative stress was proposed to be responsible for greater JNK phosphorylation in muscles from the older animals. CR has been reported to reduce oxidative stress in skeletal muscles from old rats (66) and mice (27), and such an effect may have played a role in the CR-induced decline in JNK phosphorylation in muscles from 21-mo-old mice. Levels of oxidative stress were likely not especially great in the 9-mo-old rats of the present study, consistent with the lack of significant CR-associated reductions in two indices of oxidative stress (protein carbonyls and 4-HNE protein conjugates). There is a precedent for an intervention having different effects on MAPK activation in skeletal muscle of older compared with young adult individuals. Levels of phosphorylation of JNK, p38, and ERK were greater in muscle biopsies that were sampled from 79-yr-old versus 22-yr-old men under resting conditions (64). After a strenuous resistance exercise session, phosphorylation of each MAPK was reduced compared with resting values for the older men, but the level of phosphorylation of each MAPK was either unchanged or increased for the younger men.

There were no previously published studies that determined whether skeletal muscle from CR versus AL rats differed for phosphorylation and/or abundance of mTOR, GSK-3α, or GSK3-β or for abundance of IκBα. These proteins can influence insulin sensitivity in skeletal muscle (2, 19, 31, 39, 52, 57), but the data did not support the hypothesis for CR versus AL differences for levels of phosphorylation or abundance of these signaling proteins.

Results of the present study revealed greater phosphorylation of p38 and JNK, but not ERK, in skeletal muscles from CR compared with AL rats. Skeletal muscle from CR versus AL rats did not differ for abundance of IκBα or for abundance or phosphorylation of mTOR, GSK-3α, or GSK-3β. Oxidative stress can induce insulin resistance (32), and in older animals, CR can attenuate oxidative stress in skeletal muscle (27, 66). However, two markers of oxidative stress in skeletal muscle were not different for 9-mo-old CR versus AL rats. MAPKs, GSK-3, mTOR, and IκBα can modulate insulin sensitivity, but the present results indicate that improved insulin sensitivity that is a hallmark of CR does not require altered abundance of these proteins or attenuated phosphorylation of MAPKs, mTOR, or GSK-3 in skeletal muscle.

**Perspectives and Significance**

Moderate CR by nonobese and apparently healthy adults has striking physiological effects that are associated with improved health in numerous species including rats, mice, nonhuman primates, and humans (49–51, 60, 65). Improved insulin sensitivity has been linked to many of the benefits of CR (5, 22, 51, 65). A prospective study that followed apparently healthy, nonobese, adult humans for an average of 6 yr after determination of basal insulin sensitivity provided compelling evidence that greater insulin sensitivity is coupled to improved health: there was a dose-response relationship between insulin sensitivity at baseline and clinical events during the subsequent 6 yr (21). In this context, elucidating the specific molecular mechanisms that account for well-established CR-induced elevation of skeletal muscle insulin sensitivity (16, 41, 54) has the potential for identifying new therapeutic targets for optimizing health. The results of the present study are novel and valuable because they are the first to test and to provide

<table>
<thead>
<tr>
<th>Table 4. Phosphorylated to total protein ratio in gastrocnemius</th>
<th>AL</th>
<th>CR</th>
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<tbody>
<tr>
<td>ERK1</td>
<td>1.44 ± 0.52</td>
<td>1.74 ± 0.60</td>
</tr>
<tr>
<td>ERK2</td>
<td>1.12 ± 0.18</td>
<td>2.00 ± 0.45</td>
</tr>
<tr>
<td>JNK1</td>
<td>1.03 ± 0.18</td>
<td>2.06 ± 0.48</td>
</tr>
<tr>
<td>JNK2</td>
<td>1.01 ± 0.16</td>
<td>1.92 ± 0.29*</td>
</tr>
<tr>
<td>p38</td>
<td>1.13 ± 0.21</td>
<td>3.03 ± 0.82*</td>
</tr>
<tr>
<td>mTOR</td>
<td>1.08 ± 0.11</td>
<td>1.43 ± 0.46</td>
</tr>
<tr>
<td>GSK3α</td>
<td>1.07 ± 0.14</td>
<td>1.33 ± 0.13</td>
</tr>
<tr>
<td>GSK3β</td>
<td>1.03 ± 0.17</td>
<td>1.38 ± 0.29</td>
</tr>
</tbody>
</table>

*Values are means ± SE; n = 8–12 muscles per diet group. *P < 0.05, CR greater than AL.
evidence that strongly argues against the reasonable idea that improved insulin sensitivity with CR in adult rats is attributable to attenuated activation of several important signaling pathways that can modulate insulin action. (Table 4).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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35. McCurdy CE, Davidson RT, Cartee GD. MacAulay K, Woodgett JR. McCurdy CE, Cartee GD.


