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Journal of Nutritional Biochemistry 20 (2009) 992 – 999
Journal of Nutritional Biochemistry

Chromium supplement inhibits skeletal muscle atrophy in hindlimb-suspended mice

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Received 26 July 2008; received in revised form 3 September 2008; accepted 5 September 2008

Abstract

Skeletal muscle atrophy and whole-body glucose intolerance are consequences of muscle disuse associated with conditions leading to prolonged bed rest. Nutritional supplementation with chromium has been shown to prevent weight loss and improve glucose tolerance in malnourished subjects on long-term total parenteral nutrition. The objective of this study was to evaluate the effect of oral supplementation with a novel chromium complex, chromium (D-phenylalanine)$_3$ [Cr(D-phe)$_3$] at 45 μg/kg/day for 5 weeks, on skeletal muscle atrophy and glucose intolerance in a hindlimb suspension mouse model. Hindlimb-suspended mice exhibited reduced skeletal muscle fiber size and enhanced whole-body glucose intolerance, both of which were reversed by chromium treatment. The inhibition of skeletal muscle atrophy by chromium was associated with reductions in the ubiquitination ligase atrogin-1/muscle atrophy F-box, which is elevated in hindlimb-suspended mice. Neither hindlimb suspension nor chromium treatment altered the protein levels of the myostatin, phospho-Forkhead box O-1 and mammalian target of rapamycin. Chromium-treated animals exhibited elevated Akt (*Homo sapiens* v-akt murine thymoma viral oncogene homolog) phosphorylation in their skeletal muscle, with no change observed in the levels of activated JNK (c-Jun N-terminal kinase). Thus, these data suggest that nutritional supplementation with chromium may have potential therapeutic benefits in minimizing skeletal muscle atrophy associated with long periods of muscle disuse.

Keywords: Chromium; Nutritional supplement; Insulin resistance; Skeletal muscle atrophy

1. Introduction

Prolonged immobilization or unloading of skeletal muscle causes atrophy, which is characterized by reduction in muscle cross-sectional area and compromised locomotion. Some of the conditions leading to immobilization include denervation [1], muscle unloading [2], disease states such as cachexia [3], prolonged bed rest [4,5] and advanced age [6,7]. Considerable skeletal muscle atrophy is also associated with conditions of simulated weightlessness, which is experienced by astronauts and cosmonauts during extended periods of space flight [8,9].

Although the molecular mechanisms leading to muscle atrophy are unclear, decreased protein synthesis and/or increased protein degradation is involved in the process [2,10]. Consequently, muscle atrophy may be inhibited by the augmenting signaling pathways that induce muscle growth, such as by using ectopic expression of growth factors [11,12]. However, such interventions are nonselective and possess the inherent risk of uncontrolled cell proliferation. Therefore, there is a need for newer, safer molecules that may be useful in preventing or halting the progression of muscle atrophy.

Reports from the late 1970s showed that deficiency of chromium in total parenteral nutrition (TPN) administered to long-term ill subjects can lead weight loss, severe glucose intolerance and increased insulin dependence, all of which are reversed by supplementing the TPN with chromium

Abbreviations: Akt, *Homo sapiens* v-akt murine thymoma viral oncogene homolog; ANOVA, analysis of variance; AUC, area under the curve; Cr(D-phe)$_3$, chromium (D-phenylalanine)$_3$; ELISA, enzyme-linked immunosorbent assay; Foxo, Forkhead box O; IPGTT, intraperitoneal glucose tolerance test; JNK, c-Jun N-terminal kinase; mTOR, mammalian target of rapamycin; RIPA, radioimmunoprecipitation assay; TBS, Tris-buffered saline; TPN, total parenteral nutrition.

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doi:10.1016/j.jnutbio.2008.09.006

[13,14]. Several animal and human studies have subsequently substantiated the claim that chromium is an essential nutrient required for carbohydrate and lipid metabolism (reviewed in Refs. 15–17), although some recent studies contradicted this claim [17,18].

During disuse, the muscle increasingly relies on carbohydrate metabolism for energy that eventually results in insulin resistance and whole-body glucose tolerance [19–21], indicating that insulin sensitizers may be of benefit in conditions leading to skeletal muscle atrophy. Hindlimb suspension is a commonly used animal model for studying muscle atrophy, which was originally developed as a ground-based experiment to mimic the effects of space flight on laboratory animals [22]. Several subsequent studies have demonstrated that the pattern of muscle atrophy and the biochemical changes in hindlimb-suspended rodents are similar to those observed under microgravity conditions [23,24]. Muscle atrophy during hindlimb suspension occurs in slow-twitch muscle fibers and shifts the muscle fiber type from oxidative to glycolytic subtypes [25,26]. Recent studies have shown that insulin resistance accompanies atrophy in slow-twitch skeletal muscles [27,28]. To this end, we evaluated the impact of a novel insulin sensitizer, chromium (D-phenylalanine)$_3$ [Cr(D-phe)$_3$], on muscle atrophy and whole-body glucose intolerance in a hindlimb suspension mouse model for skeletal muscle atrophy.

2. Materials and methods

2.1. Materials

Antibodies against atrogin-1/muscle atrophy F-box and myostatin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Abcam (Cambridge, MA, USA), respectively. All other primary and secondary antibodies used in the study were from Cell Signaling Technology (Boston, MA, USA). The *ACCU-CHEK* Advantage Glucose Analyzer we used was from Roche Diagnostics (IN, USA). The mouse insulin ELISA (enzyme-linked immunosorbent assay) kit was obtained from LINCO Research (St. Charles, MO, USA). Radioimmunoprecipitation assay (RIPA) buffer, protease inhibitor cocktail, phosphatase inhibitor cocktail and MicroBCA reagent and chemiluminescence reagent Super Signal West Dura Extended Duration Substrate were obtained from Pierce Biotechnology (Milwaukee, WI, USA). All other chemicals were from Sigma Chemical (St. Louis, MO, USA).

2.2. Synthesis of Cr(D-phe)$_3$

Cr(D-phe)$_3$ was synthesized and characterized as described previously [29]. Briefly, aqueous solutions of $CrCl_3.6H_2O$ (2.6 g, 10 mmol in 50 ml of water) and D-phenylalanine (4.8 g, 30 mmol in 50 ml of water) were mixed at 80°C and refluxed for 4 h. The homogeneous green reaction mixture was freeze dried. The greenish violet solid obtained was washed with acetone and dried in an air oven.

2.3. Hindlimb suspension

All experimental procedures including experimental animals were performed according to a protocol approved by the Animal Care and Use Committee of the University of Wyoming. Hindlimb suspension was performed on 12-week-old male C57/B16 mice (n=25) by randomly assigning them to either hindlimb suspension using the tail-cast model reported previously [30] or normal weight bearing/nonsuspension state. Briefly, hindlimb suspension was achieved by attaching the mice to a two-dimensional track system by their tails. This allowed the mice free access to food and water ad libitum but prevented them from placing load on their hindlimbs. Cr(D-phe)$_3$ was administered in drinking water 3 weeks prior to hindlimb suspension, and this continued for two more weeks during suspension. On the basis of calculated water intake, Cr(D-phe)$_3$ was administered to provide a dose of approximately 45 μg/kg/day (~3.8 μg of elemental chromium/kg/day). Food and water consumptions were collected twice weekly for the duration of the study and did not differ significantly between the groups. After 14 days of suspension, body weights were recorded, mice were killed by decapitation and gastrocnemius/soleus muscle, liver, heart and kidney were collected and frozen until further use.

2.4. Immunohistochemical analysis/muscle fiber cross-sectional area determination

Soleus muscles were embedded in a tissue-freezing medium and frozen in isopentane. Frozen sections (10 μm) were stained with hematoxylin and eosin to determine the fiber size. Stained sections were visualized under an Olympus IX 51 microscope equipped with a digital camera. The fiber cross-sectional area was calculated with the use of Microsuite software (Soft Imaging System, Lakewood, CO, USA). A total of 350–400 individual muscle fibers was analyzed from each group of mice.

2.5. Intraperitoneal glucose tolerance test, glucose and insulin measurements

Two days prior to euthanizing, the mice were subjected to the intraperitoneal glucose tolerance test (IPGTT) as described previously [31]. Briefly, the mice were fasted overnight (~12 h), and glucose challenge was initiated with intraperitoneal injection of glucose (2 g/kg body mass). Glucose levels were determined in blood drops obtained by clipping the tail of the mice immediately before glucose challenge, as well as at 15-, 60-, and 120-min intervals. Blood glucose levels were determined using a glucometer. Serum insulin levels were measured using a mouse insulin ELISA kit.

2.6. Insulin injection and muscle biopsy

One half of the mice in each group were injected intraperitoneally with insulin (1.5 U/100 g body mass), and the other half received saline. Ten minutes following insulin

Table 1
General features of mice treated with vehicle and those treated with chromium complex

	Control (no suspension)		Hindlimb suspension	
	Vehicle	$Cr(D\text{-phe})_3$	Vehicle	$Cr(D\text{-phe})_3$
Body mass (g)	27.3±0.33	26.8±0.43	23.7±0.16*	23.7±0.16*
Heart mass (g)	0.15±0.01	0.15±0.02	0.16±0.01	0.17±0.031
Liver mass (g)	1.40±0.03	1.42±0.04	1.24±0.07*	1.20±0.04*
Kidney mass (g)	0.34±0.02	0.32±0.05	0.43±0.03*	0.36±0.03
Glucose (mg/dl)	87.7±7.6	84.5±6.2	86.5±6.2	84.4±8.1
Insulin (ng/ml)	0.90±0.08	0.92±0.09	1.29±0.14*	0.93±0.11

* $P<05$.

injections, the animals were decapitated and skeletal muscle from their hindlimbs was quickly removed and frozen in liquid nitrogen.

2.7. Western blot analysis

Skeletal muscle tissues were rapidly removed and homogenized in ice-cold RIPA buffer containing protease and phosphatase inhibitor cocktail. Homogenates were sonicated for 15 s and centrifuged at 12,000×*g* for 20 min at 4°C. The protein concentration of the supernatant was evaluated using the MicroBCA reagent. Equal amounts (~50 μg protein/lane) of protein and prestained molecular weight markers were separated on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated for 1 h in a blocking solution containing 5% nonfat milk in Tris-buffered saline (TBS), washed in TBS and incubated overnight at 4°C with the appropriate primary antibody: anti-myostatin (1:500), anti-atrogin-1 (1:1000), anti-Akt (*Homo sapiens* v-akt murine thymoma viral oncogene homolog) (1:1000), anti-pAkt (1:1000), anti-p-JNK (c-Jun N-terminal kinase) (1:1000), anti-total JNK (1:1000) or anti-GAPDPH (1:1000). After incubation with the primary antibody, blots were incubated with an anti-rabbit IgG horseradish peroxidase-linked antibody at a dilution of 1:5000 for 1 h at room temperature. Immunoreactive bands were detected using chemiluminescence, and the intensity of the bands was quantitated with a scanning densitometer (Model GS-800; Bio-Rad) coupled with Bio-Rad PC analysis software.

2.8. Data analysis

Data are presented as the mean±S.E.M. Repeated-measures analysis of variance (ANOVA) with Bonferroni post hoc test was used to analyze the effect of time and treatment on glucose response during IPGTT (GraphPad Prism Software, San Diego, CA, USA). Total blood glucose area under the curve (AUC) was calculated for the IPGTT (time=0–120 min) using one-way ANOVA. Other comparisons were done by ANOVA followed by Fisher's least significant difference post hoc test. A p value <.05 was considered to be statistically significant.

3. Results

The general features of mice from the different treatment groups are given in Table 1. Animals subjected to hindlimb suspension had significantly lower body, heart and liver masses compared with the control mice (those that were not subjected to hindlimb suspension). In contrast, the mass of the kidney was higher in animals subjected to hindlimb suspension, which was not seen in hindlimb-suspended animals supplemented with $Cr(D\text{-phe})_3$.

Fasting blood glucose levels did not differ significantly among the control and hindlimb-suspended mice, neither did treatment with chromium have any effect on fasting blood glucose levels (Table 1). In contrast, however, serum insulin levels were significantly elevated in hindlimb-suspended mice compared with the control mice, suggesting the presence of insulin resistance. Interestingly, in mice supplemented with the chromium complex, serum insulin levels were significantly lower compared with the vehicle-treated mice (Table 1).

3.1. Effect of chromium supplementation on skeletal muscle atrophy in hindlimb-suspended mice

The muscle fibers following hindlimb suspension were analyzed for their cross-sectional area to determine if chromium supplementation had a role in maintaining muscle fiber size during disuse atrophy. Hindlimb suspension for 14 days caused a significant attenuation (~64%) in

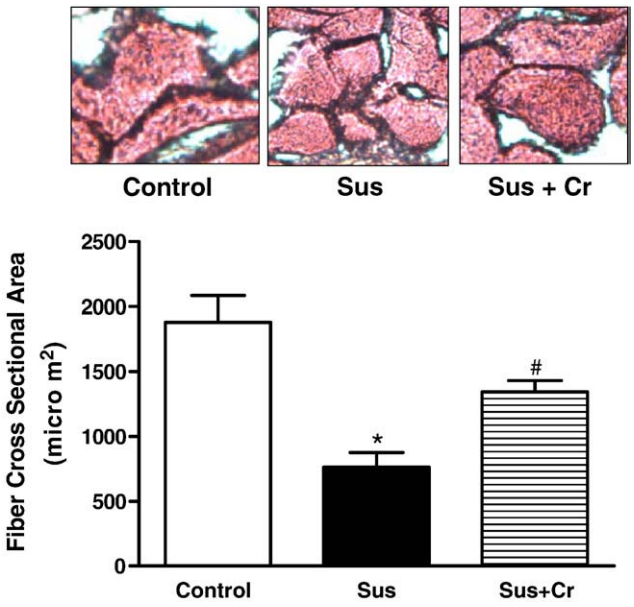

Fig. 1. Oral supplementation with $Cr(D\text{-phe})_3$ inhibits skeletal muscle fiber atrophy in mice subjected to hindlimb suspension. Representative sections from anatomically similar regions of soleus muscle from control mice (no suspension), Sus (hindlimb-suspended mice, vehicle treated) and Sus+Cr (hindlimb-suspended mice treated with chromium complex) are shown. The graph represents the fiber surface area (mean±S.E.M., n=50–70 fibers for each mice). *$P<005$ compared with nonsuspension control and vehicle-treated, suspended animals. $^{\#}P<05$ compared with nonsuspension control.

the average fiber area compared with the control mice that were not subjected to hindlimb suspension (Fig. 1). Treatment with chromium complex limited the reduction in fiber size to ~34% in hindlimb-suspended mice, suggesting that chromium treatment may cause an inhibition of loss in muscle fiber diameter that occurs during unloading. No significant difference was noted in the cross-sectional area of fibers in the control mice not subjected to hindlimb suspension that were treated with the chromium complex (data not shown).

3.2. Effect of chromium supplementation on proteins involved in maintaining skeletal muscle mass

Western blots of myostatin, atrogin-1 and phospho-Forkhead box O (Foxo)-1 transcription factors were performed to determine whether chromium treatment alters these proteins in hindlimb-suspended mice. As shown in Fig. 2A, no change was observed in the myostatin protein levels during hindlimb suspension, nor was there any alteration in myostatin levels following chromium treatment. In contrast, atrogin-1 levels were significantly elevated following 14 days of hindlimb suspension, which were significantly attenuated in mice that received chromium supplementation (Fig. 2B). Since activation of Foxo has been attributed in several forms of muscle atrophy [32,33], levels of phosphorylated Foxo-1 were determined in the muscle samples of mice subjected to hindlimb suspension. However, in contrast to previous observation, the levels of phosphorylated Foxo remained unaltered (Fig. 2C) following hindlimb suspension with or that without chromium supplementation.

3.3. Effect of chromium supplementation on whole-body glucose intolerance in hindlimb-suspended mice

Following an acute intraperitoneal glucose challenge, blood glucose levels in the control nonsuspended mice

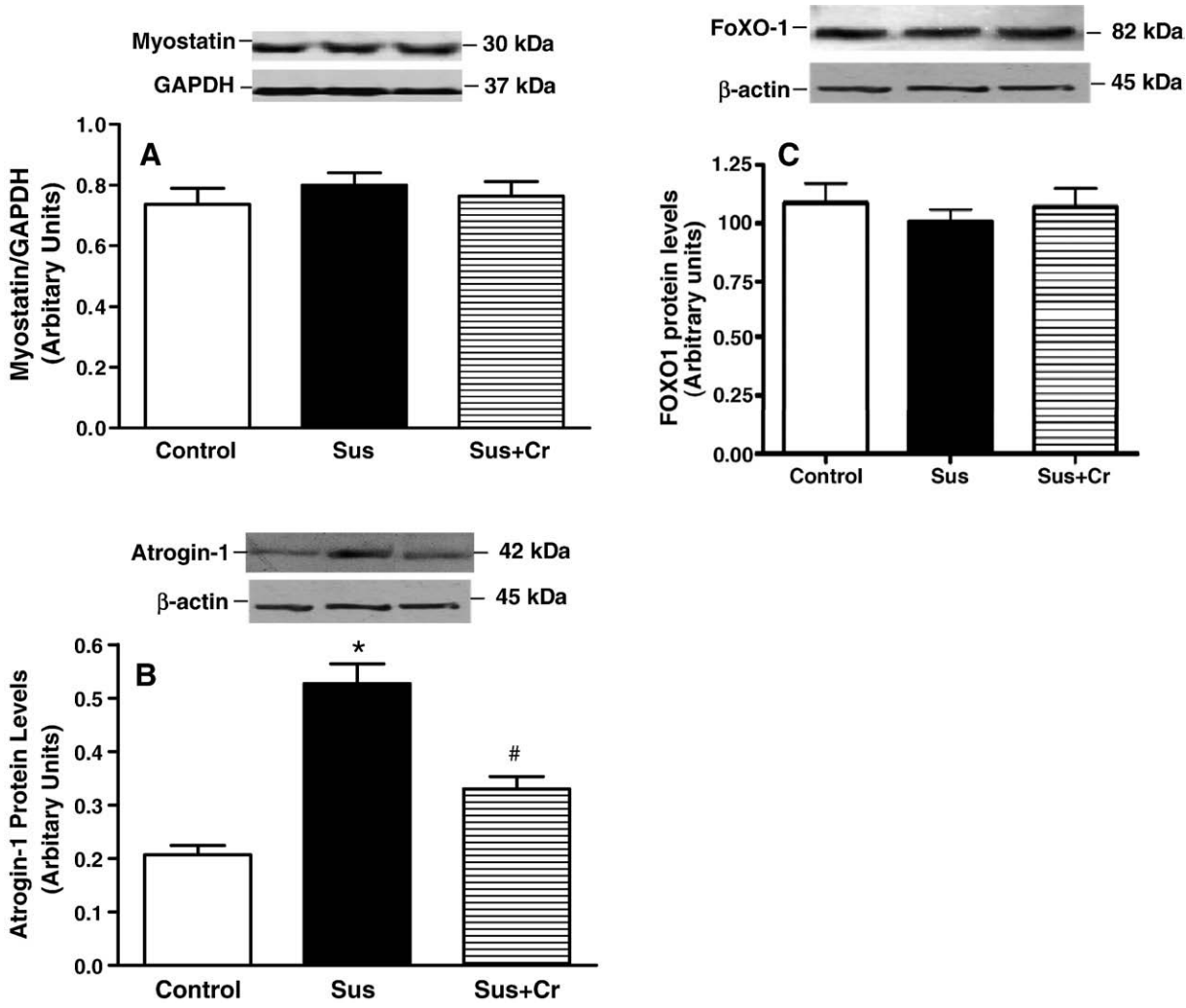

Fig. 2. Effect of oral supplementation with $Cr(D\text{-phe})_3$ on protein levels of (A) myostatin, (B) atrogin-1 and (C) FoXO1a in skeletal muscles. The upper panel is a representative Western blot (from one mice of each group), whereas the lower panel is the mean±S.E.M. of the densities of the protein bands from four to five mice of each group. *$P<.05$ compared with the control group without suspension or vehicle-fed mice subjected to hindlimb suspension. $^{\#}P<.001$ compared with mice subjected to hindlimb suspension receiving vehicle.

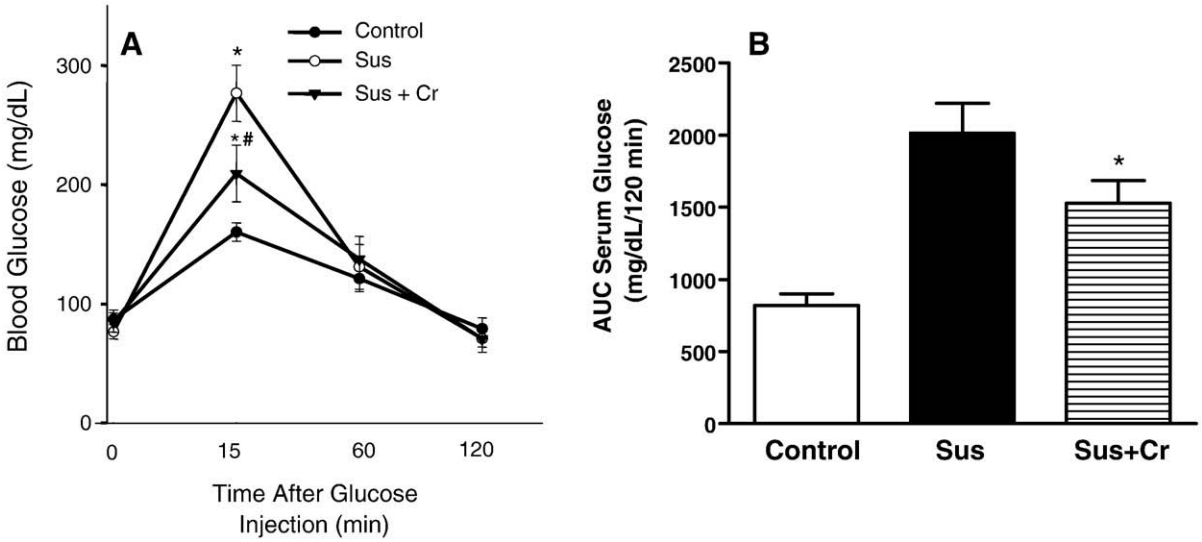

Fig. 3. Effect of oral supplementation with $Cr(D\text{-phe})_3$ on whole-body glucose tolerance test in mice subjected to hindlimb suspension. (A) Blood glucose levels were assessed in mice following glucose challenge (2 g/kg). Data are represented as the mean±S.E.M. (n=6 per group). *P<.05 vs. control group. #P<.05 vs. suspension+vehicle group. (B) AUC following glucose challenge among different groups. Data are represented as the mean±S.E.M. (n=6 per group). *P<.05 compared with the control group without suspension or vehicle-fed animals subjected to hindlimb suspension.

peaked at 15 min and began to drop thereafter, returning to near-baseline values after 60 min (Fig. 3A). In contrast, mice subjected to hindlimb suspension exhibited glucose intolerance upon glucose challenge, as evidenced by the higher postchallenge blood glucose levels. Chromium supplementation facilitated glucose clearance in hindlimb-suspended mice, with glucose disposal curves showing significantly lower blood levels of glucose at 15 min compared with the hindlimb-suspended mice. The total AUC for blood glucose was significantly higher (~1.7-fold) in the hindlimb-suspended mice compared with the control mice, which was significantly attenuated in the chromium-supplemented group (Fig. 3B).

3.4. Effect of chromium supplementation on markers of insulin signaling

Some hindlimb-suspended mice were challenged with insulin injections, following which the phosphorylation levels of Akt (Ser473) and JNK were examined in the muscle homogenates, to understand the potential mechanisms involved in the improvement in whole-body glucose utilization following chromium treatment in these mice. As anticipated, acute insulin injections resulted in an elevation of phosphorylation of muscle Akt in control mice (Fig. 4). Contrary to our expectations, the levels of phospho-Akt were higher in the muscle of hindlimb-suspended mice compared with control mice. These observations suggest that the phosphorylation status of Akt in the muscle may not be a major determinant of whole-body glucose intolerance in the hindlimb-suspended animal model. Nonetheless, in animals receiving chromium complex, the phosphorylation levels of Akt in the muscle were significantly higher than those seen in the vehicle-treated animals, suggesting that chromium may be augmenting muscle insulin signaling. In contrast to Akt, no change was observed in the phosphorylation status of JNK as a result of either suspension or chromium treatment, suggesting that

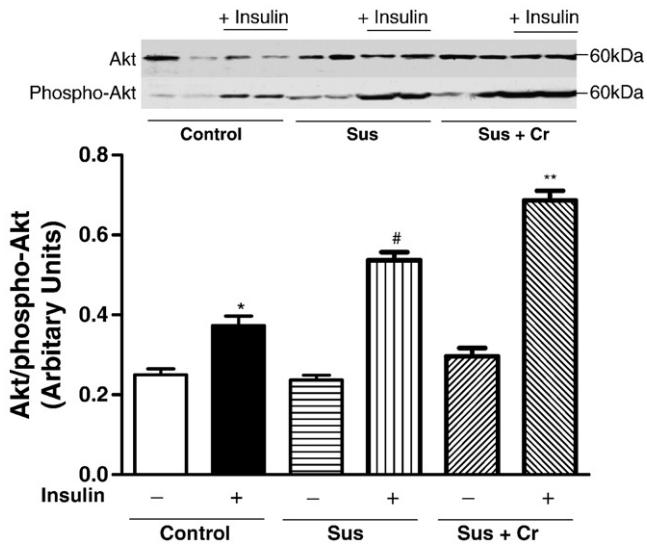

Fig. 4. Effect of oral supplementation with $Cr(D\text{-phe})_3$ on insulin-stimulated Akt phosphorylation (Ser473) in the skeletal muscle of mice subjected to hindlimb suspension. The upper panel is a representative Western blot of Akt and phospho-Akt with or without insulin stimulation in the control mice, suspended mice and suspended mice treated with chromium. The lower panel is the ratio of the densities of phospho-Akt to total Akt from all the animals in each group. Values are represented as the mean±S.E.M. (n=3 per group). *P<.05 vs. animals without insulin stimulation (control group). #P<.05 vs. control (with or without insulin). **P<.05 vs. corresponding hindlimb suspension+vehicle group (with or without insulin).

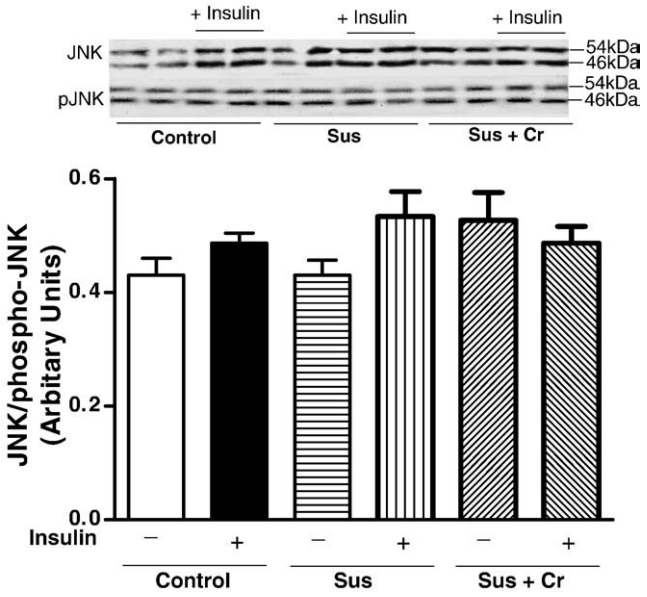

Fig. 5. Effect of oral supplementation with Cr(D-phe)$_3$ on protein levels of phospho-JNK in the skeletal muscle of mice subjected to hindlimb suspension. The upper panel is a representative Western blot for total JNK and phospho-JNK. The lower panel is the ratio of the densities of phospho-JNK to total JNK from all the animals in each group. Values are represented as the mean±S.E.M. (n=3 per group).

JNK may not be a major player in the whole-body glucose intolerance associated with hindlimb suspension (Fig. 5).

4. Discussion

The "essentiality" of chromium for glucose metabolism and insulin action was discovered accidentally when patients who were administered TNP developed symptoms similar to type 2 diabetes and weight loss, which were reversed by supplementation with chromium [13,14,34]. Based on better bioavailability and the identification that bioactive chromium exists as an oligopeptide complex, several low-molecular-weight organic chromium complexes have been designed and evaluated as potential therapeutic agents in treating insulin in type 2 diabetes [35,36]. Chromium is believed to mediate its effects by improving the binding of insulin to its receptors [37], enhancing insulin receptor kinase activity [38] and improving insulin signal transduction [39]. To our knowledge, this is the first study that examined the role of chromium supplementation on muscle atrophy. The inhibition of muscle atrophy by chromium as shown here may at least in part explain the reported reductions in weight loss associated with chromium supplementation in subjects on long-term TPN.

Although the exact mechanisms involved in skeletal muscle atrophy are not clearly known, decreased muscle growth signaling and/or increased muscle protein degradation has been postulated to be associated with muscle atrophy [2,10]. Consequently, augmenting signaling pathways that are capable of stimulating muscle growth or inhibiting muscle atrophy may ameliorate muscle atrophy. To investigate the potential mechanisms by which chromium protects against skeletal muscle atrophy, we examined the muscle protein levels of two target proteins, myostatin and atrogin-1. Myostatin is a protein that belongs to the TGF-beta family, which has been demonstrated to be a strong negative regulator of muscle growth [40]. Mice lacking the myostatin gene have been shown to develop increased skeletal muscle mass [41], whereas systemic overexpression of myostatin in mice induces muscle wasting [42]. Elevated skeletal muscle myostatin mRNA expression has been observed in rodents subjected to hindlimb unloading [43]. In the current study, however, no change was observed in the protein levels of myostatin following hindlimb suspension, nor were the levels of myostatin affected by chromium treatment. One potential explanation for this lack of effect of hindlimb suspension on myostatin may be the longer duration (14 days) for which the mice were subjected to suspension. Earlier studies have shown that myostatin mRNA peaks following 1 day of suspension but not in 3 or 7days of suspension [44]. The results of the present study tend to agree with the argument by those studies that myostatin may not represent an ideal marker for hindlimb suspension-induced muscle atrophy.

Muscle atrophy has also been associated with the enhanced mobilization of muscle proteins for degradation via ubiquitination [32]. Studies have demonstrated that atrogin-1/muscle atrophy F-box, the ubiquitin–protein ligase, is induced during early stages of atrophy and is associated with loss of muscle weight [45]. Consequently, overexpression of atrogin-1 results in myotube atrophy, whereas mice deficient in atrogin-1 are resistant to atrophy [46]. In accordance with these observations, we observed an increase in atrogin-1 protein levels in mice subjected to hindlimb suspension, which were significantly lower in mice that received chromium supplementation. Interestingly, however, we did not observe an increase in the phosphorylation of Foxo transcription factors, which have been shown to induce atrogin-1, causing atrophy [33]. Taken together, these observations suggest that chromium may be protecting against skeletal muscle atrophy by preventing the ubiquitin-mediated degradation of the muscle rather than promoting muscle growth.

Several previous studies have demonstrated the ability of chromium complexes to improve glucose utilization. However, none of the studies employed the hindlimb unloading model to investigate the effects of chromium on whole-body glucose uptake. Here, we show that oral chromium supplementation improves whole-body glucose uptake in a model of skeletal muscle disuse atrophy. The phosphoinositol-3-kinase/Akt signaling pathway is an important regulator of muscle mass [47] and is the pathway leading to the metabolic actions of insulin [48]. Since hindlimb suspension caused whole-body glucose impairment, we expected this to be associated with a reduction in muscle Akt

phosphorylation in response to insulin stimulation. Contrary to our expectations, muscles from hindlimb-suspended mice exhibited increased phosphorylation in response to insulin stimulation. We do not have a suitable explanation for this observation. Interestingly, in mice treated with the chromium complex, the extent of Akt phosphorylation in response to insulin stimulation was even higher than that seen in hindlimb-suspended mice without treatment, suggesting that the Akt signaling pathway may play a role in maintaining muscle mass. As opposed to Akt phosphorylation, the phosphorylation and activation of JNK serve as negative feedback regulators of insulin action by phosphorylating serine on insulin receptor substrate, resulting in decreased insulin-stimulated tyrosine phosphorylation, Akt phosphorylation and glucose uptake [49]. The lack of effect of hindlimb suspension or chromium treatment on JNK phosphorylation levels suggests that the JNK pathway may not be involved in the regulation of skeletal muscle mass. The mammalian target of rapamycin (mTOR), which is downstream of Akt, has been implicated in muscle mass maintenance — reduced levels of mTOR activity have been associated with muscle atrophy, whereas elevated levels of mTOR have been shown to increase muscle mass [47]. Similar to JNK, muscle mTOR levels were not altered following hindlimb suspension, nor with treatment with chromium (figure not shown).

In summary, the present study shows that oral supplementation with chromium attenuates disuse-induced atrophy of skeletal muscle fibers and improves whole-body glucose utilization in a mouse hindlimb suspension model. The atrophy-related protein atrogin-1 that was elevated in the muscle of mice following hindlimb suspension was attenuated in mice that received chromium complex. No alteration in myostatin levels was seen in response to chromium treatment. Chromium also augmented insulin-induced phosphorylation of Akt in the muscle without altering JNK levels. Taken together, these results suggest that oral supplementation with chromium may have beneficial effects in treating skeletal muscle atrophy resulting from long periods of muscle disuse.

Acknowledgments

This work was supported by grants from the American Diabetes Association (Junior Faculty Award) and Wyoming NASA Space Grant Consortium to N.S. We thank Dr. Scott M. Smith (Nutritional Biochemistry Human Adaptation and Countermeasures Division, NASA Johnson Space Center, Houston, TX) for useful discussion and Mr. Machender Reddy (School of Pharmacy, University of Wyoming) for technical assistance.

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