

Chromium (D-phenylalanine)₃ Supplementation Alters Glucose Disposal, Insulin Signaling, and Glucose Transporter-4 Membrane Translocation in Insulin-Resistant Mice^{1–3}

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Abstract

Chromium has gained popularity as a nutritional supplement for diabetic and insulin-resistant subjects. This study was designed to evaluate the effect of chronic administration of a novel chromium complex of D-phenylalanine [Cr(D-phe)₃] in insulin-resistant, sucrose-fed mice. Whole-body insulin resistance was generated in FVB mice by 9 wk of sucrose feeding, following which they were randomly assigned to be unsupplemented (S group) or to receive oral Cr(D-phe)₃ in drinking water (SCr group) at a dose of 45 $\mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (~3.8 μg of elemental chromium $\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). A control group (C) did not consume sucrose and was not supplemented. Sucrose-fed mice had an elevated serum insulin concentration compared with controls and this was significantly lower in sucrose-fed mice that received Cr(D-phe)₃, which did not differ from controls. Impaired glucose tolerance in sucrose-fed mice, evidenced by the poor glucose disposal rate following an intraperitoneal glucose tolerance test, was significantly improved in mice receiving Cr(D-phe)₃. Chromium supplementation significantly enhanced insulin-stimulated Akt phosphorylation and membrane-associated glucose transporter-4 in skeletal muscles of sucrose-fed mice. In cultured adipocytes rendered insulin resistant by chronic exposure to high concentrations of glucose and insulin, Cr(D-phe)₃ augmented Akt phosphorylation and glucose uptake. These results indicate that dietary supplementation with Cr(D-phe)₃ may have potential beneficial effects in insulin-resistant, prediabetic conditions. *J. Nutr.* 138: 1846–1851, 2008.

Introduction

Diabetes is a major health concern that has reached pandemic proportions. The number of people with diabetes worldwide is anticipated to double from the current estimate of 150 million to ~300 million in 2025 (1). Metabolic syndrome, characterized by a cluster of conditions such as glucose intolerance, hypertension, dyslipidemia, inflammation, and obesity, is thought to be the prediabetic state that predisposes individuals to full-blown diabetes (2,3). Although the etiology of metabolic syndrome is unclear, insulin resistance, defined as a state of impaired biological response to normal or elevated serum insulin concentration, appears to be the primary pathology. Epidemiologic data indicate that the prevalence of metabolic syndrome has steadily increased over the past 2 decades, underscoring the need for successful strategies for treating and/or preventing this condition (4).

Besides genetic makeup, a sedentary lifestyle and a high-energy western diet are thought to be the primary causes of metabolic syndrome. Consequently, lifestyle changes such as diet, exercise, and weight management have been tried as non-pharmacological interventions (5). As time progresses, lifestyle changes alone become insufficient and further intervention with insulin-sensitizing drugs such as the thiazolidinediones are necessary. These drugs, although they prevent disease progression by improving carbohydrate metabolism, are associated with several adverse effects that limit their use. Therefore, there has been substantial demand for the development of newer agents to improve overall insulin sensitivity and provide long-term benefit in the management of metabolic syndrome. In this context, the design and characterization of effective and safe nutritional supplements that can alleviate insulin resistance represents an attractive strategy to counter metabolic syndrome (6).

The mineral chromium, formulated either alone or as an ingredient of multivitamin formulations or fortified-food such as breakfast cereal, ranks next only to calcium in sales among the mineral supplements in the market. Dietary deficiency of chromium has been shown to be positively associated with the risk of diabetes and its complications (7,8). Consequently, dietary supplementation with chromium has been shown to lower blood glucose concentrations and improve lipid profile in

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diabetic patients (9). These effects of chromium are thought to be mediated by its ability to increase insulin binding to its receptor (10), increase the number of insulin receptors (11), and enhance insulin receptor kinase activity (12). In a recent clinical trial, Martin et al. (13) demonstrated improved insulin sensitivity in subjects with type 2 diabetes treated with chromium. In contrast, however, some reports claim that chromium treatment may not have any benefit in diabetic subjects (14,15). Further studies are therefore necessary to address these controversies and to understand the potential role of chromium in treating insulin resistance.

Based on the better bioavailability and the identification that biologically active chromium is an oligopeptide complex, several low-molecular weight (LMW)⁶ organic chromium complexes have been designed and evaluated as potential therapeutic agents to treat insulin resistance. The present study evaluated the impact of supplementation with a novel chromium complex of the amino acid D-phenylalanine [Cr(D-phe)₃] (16) on whole-body glucose tolerance and skeletal muscle insulin signaling in a dietary, sucrose-fed, insulin-resistant mouse model. The study also assessed the in vitro effect of chromium on insulin signaling and glucose uptake in insulin-resistant cultured adipocytes.

Materials and Methods

Materials. All chemicals unless stated otherwise were obtained from Sigma Chemical. The Micro BCA protein assay kit was purchased from Pierce Chemical.

Synthesis of Cr(D-phe)₃. Cr(D-phe)₃ was synthesized and characterized as described previously (16). Briefly, aqueous solutions (50 mL each) of CrCl₃·6H₂O (2.6 g, 10 mmol) and D-phenylalanine (4.8 g, 30 mmol) 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.

Mouse treatment protocol. This study was performed via a protocol approved by the Institutional Animal Care and Use Committee of the University of Wyoming in accordance with the Guide for the Care and Use of Laboratory Animals. Care was taken to minimize discomfort, distress, and pain to the animals. Three-month-old male FVB mice were bred in our research vivarium and housed in a controlled environment (12-h-daylight cycle) in groups of 5 mice per cage at 22°C. The mice consumed water and a purified, high-starch diet (C) (68% of energy from corn starch, 20% protein, 12% fat; D11724, Research Diets) ad libitum for 1 wk. After the baseline period, mice (*n* = 22) were randomly assigned to either continue receiving diet C or were fed a high-sucrose diet (S) (68% of energy from sucrose, 20% protein, 12% fat; D11725, Research Diets) for 9 wk (Table 1). One-half of the mice consuming the S diet were randomly assigned to receive Cr(D-phe)₃ (SCr group) via drinking water for 9 wk. Based on the calculated water intake, Cr(D-phe)₃ was administered to provide a dose of ~45 μg·kg⁻¹·d⁻¹, corresponding to ~3.8 μg of elemental chromium·kg⁻¹·d⁻¹. The drinking water containing the chromium supplement was freshly prepared every other day.

Intraperitoneal glucose tolerance test, blood glucose, and serum insulin. Two days prior to killing, mice were subjected to the intraperitoneal glucose tolerance test (IPGTT) as described previously (17). Briefly, food was withheld from the mice overnight (~12 h) and glucose challenge was initiated with an intraperitoneal injection of

TABLE 1 Composition of the diets used in the experiments

Ingredients	Control diet	Sucrose diet
	g/kg	
Casein, 80 mesh	200	200
Corn starch	500	0
Maltodextrin 10	150	0
Sucrose	0	650
Dl-Methionine	3	3
Corn oil	50	50
Cellulose	50	50
Mineral mix ¹	500	500
Vitamin mix ²	24	24

¹ Mineral mix, S10001 composition (g/kg): calcium phosphate, dibasic, 500; magnesium oxide, 24; potassium citrate, 220; potassium sulfate, 52; sodium chloride, 74; chromium potassium sulfate 0.55; cupric carbonate, 0.3; potassium iodate, 0.01; ferric citrate, 6; manganous carbonate, 3.5; sodium selenite, 0.01; zinc carbonate, 1.6.

² Vitamin mix, V10001 composition (g/kg): vitamin A palmitate (synthetic vitamin A all trans retinol acetate), 0.8; cholecalciferol, 1.0; vitamin E acetate (d,l-alpha-tocopherol acetate), 10.0; menadione sodium bisulfate, 0.08; biotin 1%, 2.0; cyanocobalamin 0.1%, 1.0; folic acid, 0.2; nicotinic acid, 3; calcium pantothenate, 1.6; pyridoxine-HCl, 0.7; riboflavin, 0.6; thiamin-HCl, 0.6.

glucose (2 g/kg). Glucose concentrations were measured 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 concentrations were measured using ACCU-CHEK Advantage Glucose Analyzer (Roche Diagnostics). Serum insulin concentrations were measured using a mouse insulin ELISA kit (LINCO Research).

Insulin injection and isolation of skeletal muscle. One-half of the mice in each group were injected intraperitoneally with insulin (1.5 U/kg body mass) and the other one-half received the vehicle. Ten minutes following insulin injection, the mice were killed by decapitation. The skeletal muscle from hind legs was quickly removed, frozen in liquid nitrogen, and stored at -80°C until used.

Skeletal muscle membrane protein extraction. Skeletal muscle membrane protein was extracted using a membrane protein extraction kit (Biovision). The membrane protein was subsequently used for Western blot analysis of glucose transporter-4 (GLUT4).

Western-blot analysis of GLUT4, p-c-Jun N-terminal kinases, and total c-Jun N-terminal kinases, p-AKT, total AKT, and insulin receptor. Skeletal muscles tissue were rapidly removed, homogenized, and subjected to Western blot analysis as described previously (16). Wherever indicated, blots were stripped and reprobed with antibodies directed at the total protein. The intensity of bands was measured with a scanning densitometer (Model GS-800; Bio-Rad) coupled with Bio-Rad PC analysis software.

Cell culture and treatment. 3T3-L1-pre-adipocytes were obtained from the American Type Culture repository and differentiated to adipocytes in the presence of 0.2 mg/L dexamethasone, 0.5 mmol/L 1-isobutyl-3-methylxanthine, and 10 mg/L insulin as reported previously (16). Quiescent cells were rendered insulin resistant by treating them for 24 h with either 1 nmol/L insulin and/or 10 mmol/L glucose (18). Following treatment, cells were washed and incubated with fresh media (without serum or insulin) for 2 h, following which they were transiently (5–10 min) stimulated with insulin (10 nmol/L).

Cellular glucose-uptake assay. 2-Deoxy-D-[³H] glucose deoxyglucose uptake into 3T3-L1 adipocytes was performed as described previously (16).

Data analysis. Data are presented as means ± SEM. We used repeated-measures ANOVA with Bonferroni post hoc tests to analyze the effects of time and treatment on glucose responses during IPGTT (GraphPad Prism Software). Total blood glucose area under the curve (AUC) was

⁶ Abbreviations used: AUC, area under the curve; C, control group; Cr(D-phe)₃, chromium complex of D-phenyl-alanine; GLUT4, glucose transporter-4; IPGTT, intraperitoneal glucose tolerance test; IRS-1, insulin-receptor substrate-1; JNK, c-Jun N-terminal kinase; LMW, low-molecular weight; S, sucrose-treated; SCr, sucrose + Cr(D-phe)₃.

calculated for the IPGTT (time 0–120 min) and analyzed using a 1-way ANOVA. Statistical evaluations of data in Figures 2, 3, and 4 were performed using 2-way ANOVA and Scheffé post-hoc analysis (SAS/STAT version 6, SAS Institute). Data in Figures 5 and 6 were analyzed by 1-way ANOVA followed by the Bonferroni multiple comparisons post hoc test. A *P*-value of <0.05 was considered significant.

Results

Body mass, blood glucose, and serum insulin. Body, heart, liver, and kidney masses and organ:body mass ratios did not differ among the C mice and the 2 groups of sucrose-fed mice (Table 2). Consistent with previous findings (19,20), serum insulin concentrations were significantly elevated in S mice compared with the C mice, suggesting insulin resistance. The SCr group had significantly lower serum insulin concentrations than the S group and did not differ from the C mice. In contrast, blood glucose concentrations following food deprivation did not differ among the 3 groups (Table 2).

IPGTT. Following acute intraperitoneal glucose challenge, blood glucose concentrations in C mice began to drop after peaking at 15 min and returned to nearly baseline values after 120 min (Fig. 1A). In contrast, S mice exhibited severe hyperglycemia upon administration of glucose and exhibited impaired glucose tolerance as evidenced by the high postchallenge blood glucose concentrations. Chromium supplementation facilitated glucose clearance in sucrose-fed mice with significantly lower blood concentrations of glucose at 15 and 60 min compared with the sucrose-fed mice. The total AUC for blood glucose was significantly higher in the sucrose-fed mice compared with controls, which did not differ from the SCr group (Fig. 1B).

Skeletal muscle insulin signaling. Baseline levels of phospho-Akt in skeletal muscle homogenates did not differ among the 3 groups of mice (Fig. 2). As anticipated, insulin injection significantly increased Akt phosphorylation in the skeletal muscle of the C group. In contrast, sucrose-fed mice responded poorly to insulin challenge as evidenced by the lower levels of phospho-Akt compared with insulin-treated controls. In contrast, Akt phosphorylation did not differ from controls in sucrose-fed mice supplemented with the chromium complex (Fig. 2). However, the phosphorylation status of insulin receptor did not differ among the groups under basal or insulin-stimulated conditions (Supplemental Fig. 1).

The amount of GLUT4 translocated to the plasma membrane were significantly higher in the skeletal muscle of insulin-

TABLE 2 Blood and organ masses, blood glucose, and serum insulin of 9-wk-old male C, S, and SCr mice¹

Mouse group	C	S	SCr
<i>n</i>	6	8	8
Body mass, g	28.9 ± 1.6	30.4 ± 0.7	29.1 ± 1.2
Heart mass, g	0.17 ± 0.01	0.16 ± 0.01	0.16 ± 0.01
Liver mass, g	1.47 ± 0.07	1.39 ± 0.07	1.31 ± 0.09
Kidney mass, g	0.47 ± 0.04	0.43 ± 0.03	0.42 ± 0.05
Blood glucose, ² mmol/L	5.43 ± 0.41	6.02 ± 0.37	5.78 ± 0.24
Serum insulin, ² pmol/L	138.3 ± 20 ^a	241.6 ± 28 ^b	158.3 ± 20 ^a

¹ Values are means ± SEM. Means in a row with superscripts without a common letter differ, *P* < 0.05.

² Food-deprived mice were sampled.

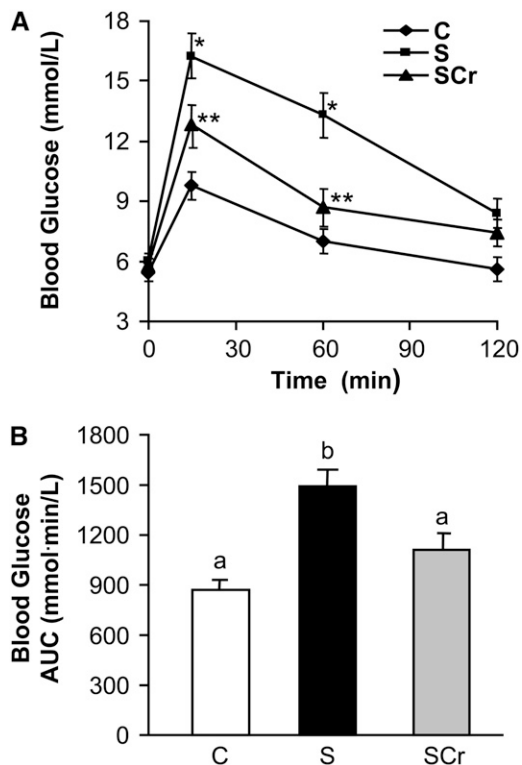


FIGURE 1 Blood glucose concentrations (A) and incremental AUC (120 min) in C, S, and SCr mice following a glucose challenge (2 g/kg). Values are means ± SEM, *n* = 6–8. A: *Different from C at that time, *P* ≤ 0.05; **different from S at that time, *P* < 0.05. B: Means without a common letter differ, *P* < 0.05.

injected C mice compared with uninjected mice (Fig. 3). In contrast, insulin stimulation did not increase the concentrations of membrane-associated GLUT4 in the muscles of sucrose-fed mice. As with Akt phosphorylation, a near-complete recovery, to a level that did not differ from insulin-stimulated controls, occurred in membrane-associated GLUT4 concentrations in muscle of SCr mice (Fig. 3).

c-Jun N-terminal kinases (JNK) are activated by inflammatory cytokines and are thought to play a key role in the pathophysiology of insulin resistance through negative regulation of insulin signaling (21). In the sucrose-fed mice, however, JNK phosphorylation did not differ from controls under basal or insulin stimulated conditions (Fig. 4). Treatment with Cr(D-phe)₃ also did not affect JNK concentrations.

Insulin resistance in cultured adipocytes. Cultured adipocytes chronically subjected to high insulin and glucose concentrations were insulin resistant as indicated by the inhibition of Akt phosphorylation in response to transient insulin (0.01 mmol/L, 5–10 min) challenge (Fig. 5). Pretreatment with chromium inhibited the attenuation of Akt-phosphorylation in insulin-resistant cells. Interestingly, at higher concentrations, Cr(D-phe)₃ augmented insulin-stimulated phosphorylation of Akt in insulin-resistant cells to levels beyond that of control cells (Fig. 5).

Chronic treatment with insulin and glucose caused a reduction in insulin-stimulated glucose uptake in 3T3-adipocytes, which, like skeletal muscle cells, use GLUT4 as glucose transporter (Fig. 6). Treatment with Cr(D-phe)₃ reversed the blunting of glucose uptake induced by high insulin and glucose concentrations. Similar to its effects on Akt phosphorylation, at higher concentrations, glucose uptake in the Cr(D-phe)₃-treated cells was

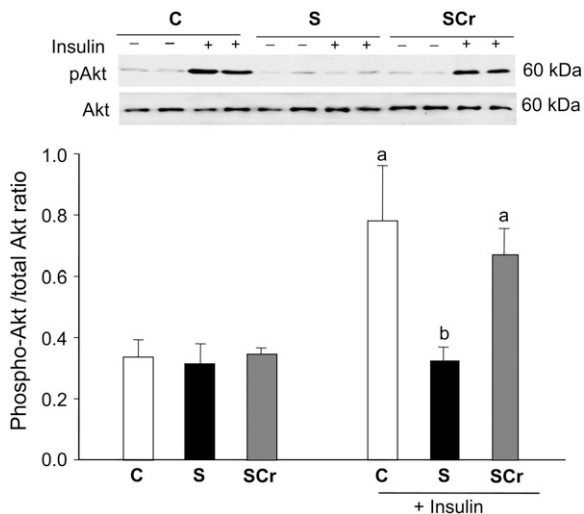


FIGURE 2 Akt phosphorylation in unstimulated and stimulated skeletal muscle from C, S, and SCr mice. The upper panel is a representative Western blot of phospho-Akt and pan-Akt in muscle with or without insulin stimulation. The lower panel is the ratio of the densities of phospho-Akt:total Akt. Values are mean \pm SEM, $n = 4-6$. Labeled means without a common letter differ, $P < 0.05$.

significantly higher than that of control cells not subjected to insulin-resistant conditions.

Discussion

The major finding from the present study is that chronic oral supplementation with Cr(D-phe)₃ improved glucose tolerance in a dietary, sucrose-fed mouse model of insulin resistance. Skeletal muscle from Cr(D-phe)₃-treated mice had enhanced insulin-stimulated Akt-phosphorylation and membrane translocation of GLUT4. In vitro studies showed that Cr(D-phe)₃ augmented

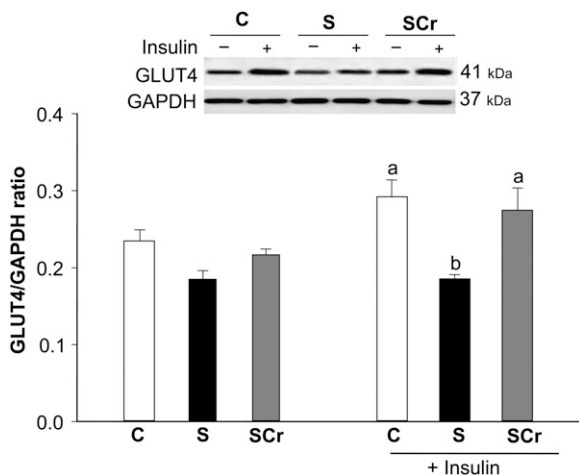


FIGURE 3 Membrane GLUT4 translocation in unstimulated and stimulated skeletal muscle from C, S, and SCr mice. The upper panel is a representative Western blot of membrane associated GLUT4 with or without insulin stimulation. Glyceraldehyde 3-phosphate dehydrogenase was used as a loading control. The lower panel is the mean density of the GLUT4 band from all the mice used in the study. Values are mean \pm SEM, $n = 4-6$. Labeled means without a common letter differ, $P < 0.05$.

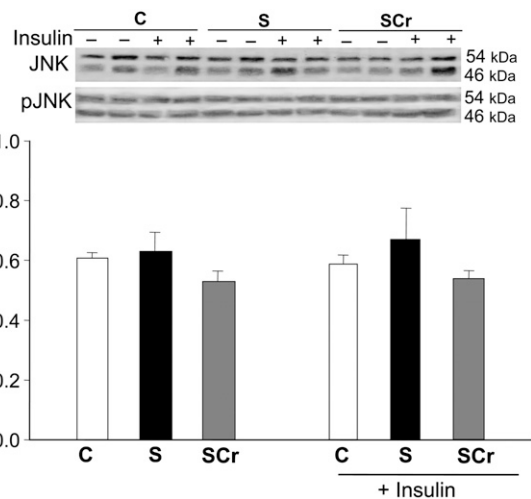


FIGURE 4 JNK phosphorylation in unstimulated and stimulated skeletal muscle from C, S, and SCr. The upper panel represents Western blots for total and phosphorylated JNK protein concentrations. The lower panel is the ratio of the densities of phospho-JNK:total JNK. Values are mean \pm SEM, $n = 4-6$.

insulin-stimulated Akt-phosphorylation and glucose uptake in cultured adipocytes rendered insulin resistant by subjecting them to high insulin and glucose concentrations. Taken together, these results suggest that oral supplementation with Cr(D-phe)₃ may have potential beneficial effects in the treatment of insulin resistance.

Studies during the last decade suggest that elemental chromium (III) may play an essential role in carbohydrate and lipid metabolism (20). The biologically active form of chromium was identified as an oligopeptide chromodulin, a LMW chromium-binding substance (22). Based on these findings, LMW chromium complexes have been synthesized and evaluated as insulin-potentiating agents. Among the chromium complexes used, chromium picolinate has gained popularity as a nutritional supplement. However, there have been some concerns regarding

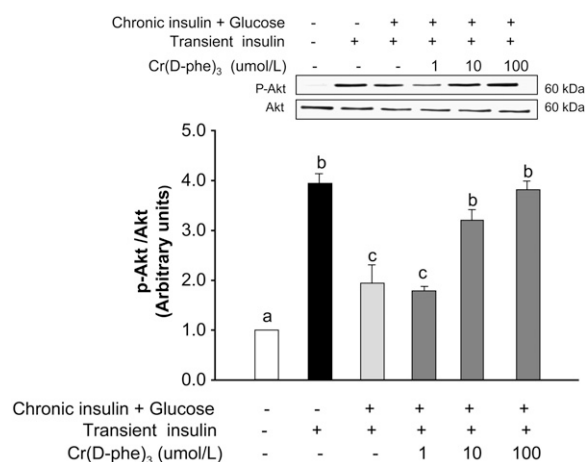


FIGURE 5 Akt phosphorylation in insulin-resistant adipocytes either the presence or absence of Cr(D-phe)₃. The upper panel is a representative Western blot of phospho-Akt and pan-Akt in muscle with or without acute insulin stimulation. The lower panel is the ratio of the densities of phospho-Akt:total Akt. Values are mean \pm SEM, $n = 3$. Means without a common letter differ, $P < 0.05$.

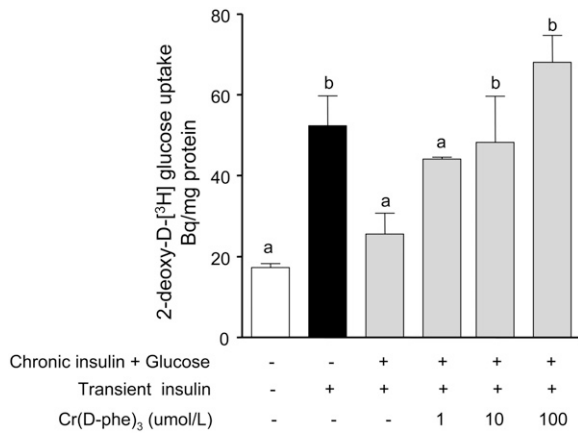


FIGURE 6 Glucose uptake in insulin-resistant adipocytes in either the presence or absence of Cr(D-phe)₃. Values are mean ± SEM, *n* = 3. Means without a common letter differ, *P* < 0.05.

the mutagenic potential of chromium picolinate (23), which has been attributed to oxidative stress caused by the picolinate ligand (24). Cr(D-phe)₃ used in the present study was designed to mimic the activity of chromodulin, which is an oligopeptide complex of chromium with 4 amino acids. Compared with the picolinate ligand, the phenylalanine ligand used in the current study has better solubility at physiological pH and may also inhibit oxidative stress (25). Derivatives of D-phenylalanine, such as nateglinide, have been shown to have beneficial effects in type II diabetes, which may represent an additional advantage of using this ligand (26). Another reason to use the D-isomer rather than the naturally available L-isomer is to have better bioavailability by delaying the metabolism of the D-amino acid.

Previous studies have shown that supplementation with chromium improves insulin signaling and glucose intolerance in genetic models of type 2 diabetes (16,27). Wang et al. (16,27,28) demonstrate that chromium supplementation to the insulin-resistant JRC:LA-corpulent rat at a dose of 80 μg·kg⁻¹·d⁻¹ improved glucose disposal rates and significantly increased insulin-stimulated phosphorylation of insulin receptor substrate-1 and phosphatidylinositol-3 kinase activity in skeletal muscles compared with control rats. Yang et al. (16) demonstrated that Cr(D-phe)₃ given orally at 150 μg·kg⁻¹·d⁻¹ improves glucose tolerance and insulin signaling in leptin-deficient, obese mice. Clodfelder et al. (28) reported that oral administration of a LMW chromium propionate complex improves insulin sensitivity in Zucker diabetic obese rats. Whereas most of these models represented the utility of chromium complex in a genetic model of diabetes, the present study shows that chromium can alleviate insulin resistance in a dietary model of insulin resistance.

The mechanism by which chromium imparts its beneficial effects is yet unclear, although several hypotheses have been proposed. The biologically active chromium complex, chromodulin, has been shown to augment kinase activity of the insulin receptor in adipocytes by over 8-fold (29). Chromium improves glucose uptake (27) and enhances the translocation of GLUT4 to the cell membrane (30) in cultured adipocytes. It has also been suggested that chromium can attenuate the concentrations of protein tyrosine phosphatase 1B, which dephosphorylates the insulin receptor and functions as a negative regulator of insulin signaling (27). This study shows that chronic supplementation with chromium improves the insulin-stimulated phosphorylation of Akt and membrane translocation of GLUT4 in skeletal

muscle of insulin-resistant mice, supporting the observations that chromium may act by augmenting insulin signaling. However, no change in phosphorylation status of insulin receptor occurred in the skeletal muscle (Supplemental Fig.1), which contradicts the aforementioned reports. Although the reason for this discrepancy is unknown, it is likely that the transient nature of the phosphorylation of insulin receptor may have precluded its detection in the experiments described here.

Phosphorylation and activation of JNK can lead to serine phosphorylation of insulin-receptor substrate-1 (IRS-1) that prevents tyrosine phosphorylation of IRS-1 in response to insulin stimulation (31). IRS-1-phosphorylated at the serine residue also undergoes rapid ubiquitination, causing blunting of insulin signaling (32). Thus, serine phosphorylation of JNK represents a key signaling pathway that mediates insulin resistance. In the sucrose-fed, insulin-resistant mouse model, however, insulin resistance was not associated with JNK phosphorylation. Neither did chromium treatment alter the concentrations of JNK phosphorylation. These results suggest that JNK may not play a major role in insulin resistance induced by chronic sucrose feeding.

Taken together, the results from this study show that a new chromium complex alleviates diet-induced insulin resistance, which may be mediated by augmenting insulin signaling. These results suggest that nutritional supplementation with chromium complexes may have potential therapeutic value in treating or preventing insulin resistance associated with metabolic syndrome.

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