Insulin-sensitizing and cholesterol-lowering effects of chromium (D-Phenylalanine)₃

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Abstract

Low-molecular weight organic chromium complexes are thought to play a key role in carbohydrate and lipid metabolism and therefore have been gaining popularity as nutritional supplement for patients with diabetes and concomitant lipid disorders. The aim of the present study was to evaluate the effects of a novel synthetic chromium (D-phenylalanine)₃ complex on insulin-sensitivity, plasma lipid-profile and oxidant stress in a mouse model of type II diabetes. Plasma glucose levels following intraperitoneal insulin-challenge (1 U/kg) to obese ob/ob (+/+ ) mice treated with Cr(D-Phe)₃ (150 μg/kg/day for 6 weeks) were significantly lower compared to vehicle-control (control: 175.8 ± 43.2 mg/dL versus Cr(D-Phe)₃ 115.3 ± 18.0 mg/dL, p < 0.01, n = 12). Total serum cholesterol to high-density lipoprotein ratio was significantly reduced following Cr(D-Phe)₃ treatment (control: 2.19 ± 0.08 versus Cr(D-Phe)₃ 1.63 ± 0.05; p < 0.05). Hepatic oxidant stress, assessed as malondialdehyde equivalents and protein-carbonyl content were significantly attenuated following Cr(D-Phe)₃ treatment. The complex also inhibited lipid-peroxidation in vitro, in a concentration dependent manner. Taken together, these data suggest that Cr(D-Phe)₃ may be of potential value in the therapy or prophylaxis of insulin-resistance and dyslipidemia associated with obesity.

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1. Introduction

Insulin resistance, concomitant with type II diabetes, obesity, hypertension, and other features of the metabolic syndrome is the major risk factor for cardio-vascular diseases and one of the leading causes of mortality and morbidity [1]. Proper management of insulin resistance (with cardiovascular drugs as well as non-drug therapy such as exercise, caloric restriction) plays a pivotal role in reducing the risk for cardiovascular diseases. However, many drugs targeted for insulin resistance are often complicated with undesired effects which may compromise their ultimate clinical efficacy. Chromium is thought to play a key role in normal carbohydrate and lipid metabolism by potentiating the action of insulin [2]. Clinical trials have demonstrated that dietary chromium supplementation can lower blood glucose levels and improves lipid profile in diabetic patients [3,4].

Better bioavailability of low-molecular-weight (LMW)-organic chromium complexes and the identification that the biologically active form of chromium is a complex with an oligopeptide, prompted the design and evaluation of LMW-organic chromium complexes as therapeutic agents to counter the diminished effect of insulin in type-II diabetics [5,6]. Chromium complex of picolinic acid, the most popularly used dietary supplement has been shown to modulate intracellular pathways of glucose metabolism and improve comorbidities associated with insulin resistance in several animal and human studies [7]. However, recent reports that chromium picolinate may cause deleterious effects on DNA [8] through generation of oxygen radicals, greatly limits its therapeutic utility.
Recent studies from our lab and those from others suggest that chromium complexes of amino acids may be safer and efficacious alternatives to the commercially available chromium picolinate [9–11]. Anderson et al., have shown that chromium(III)–histidinate complexes exhibits increased stability and bioavailability compared to other commercially available chromium supplements [12]. This complex was also devoid of the DNA-nicking properties attributed to the commercially available chromium picolinate [11]. We have recently reported that a novel chromium (d-phenylalanine)3 [Cr(d-Phe)3] complex improves insulin-signal transduction and glucose uptake in cultured adipocytes [9]. Cr(d-Phe)3 also caused a marked improvement in glucose tolerance in obese mice [9]. Like chromium–histidinate, Cr(d-Phe)3 did not cause DNA-damage under physiological conditions [9]. These studies suggest that chromium–amino acid complexes may serve as better alternatives to the chromium complexes that are currently used as dietary supplements.

Based on the aforementioned considerations the aim of the present study was to investigate the effect of Cr(d-Phe)3 on insulin-sensitivity, serum lipid profile and oxidative stress in a mouse model of type-II diabetes.

2. Materials and methods

2.1. Materials

All antibodies used in this study were from Cell Signaling Technology Inc. (Beverly, MA). Micro BCA protein assay kit was from Pierce Chemical (Rockford, IL). Kits for triglyceride, total cholesterol and high density lipoprotein (HDL) were from Equal Diagnostics (Exton, PA). All other chemicals were from Sigma-Aldrich Chemical Co (St. Louis, MO).

2.2. Synthesis of Cr(d-Phe)3

Cr(d-Phe)3 was synthesized and characterized as described previously [9]. Briefly, aqueous solutions of CrCl3 • 6H2O (2.6 g; 10 mmol in 50 mL water) and d-phenylalanine (4.8 g, 30 mmol in 50 mL 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 air oven.

Based on the stoichiometry, elemental analysis and spectral studies, the product obtained is a complex containing chromium and phenylalanine in a ratio of 1:3 with the proposed structure as shown in Fig. 1.

2.3. Animals and treatment protocol

All animal treatment procedures described in this study were approved by the Animal Care and Use Committee at University of Wyoming (Laramie, WY). Homozygous B6.Vlepb(+/+) male mice purchased from the Jackson Laboratory (Bar Harbor, ME) at age 5 weeks were divided randomly into two weight-matched groups, marked as ob/ob(+/+) control and ob/ob(+/+) treatment (n = 12). Number and age-matched normal C57 mice were used as lean control. All animals were maintained on conventional laboratory diet under well-controlled conditions of temperature (22 ± 2 °C), humidity (55 ± 5%) and 12 h/12 h light-dark cycle and had ad libitum access to water and standard rodent chow. Cr(d-Phe)3 was provided in the drinking water and, on the basis of water intake, was administered to provide an intake of about 150 μg/kg/day corresponding to about 10–15 μg elemental Cr/kg/day for ob/ob(+/+) and lean treatment groups [9]. One set of mice was used for the insulin-challenge test whereas the other set of mice, which did not receive insulin was fasted overnight and sacrificed by cerebral dislocation. The livers of these mice were frozen in liquid nitrogen immediately and stored at −80 °C until use. Blood was collected from the heart, and the serum was extracted by centrifuging the blood at 1000 g at 4 °C and stored at −80 °C. Weights of body and other organs were measured with a standard laboratory scale.

2.4. Intraperitoneal insulin-tolerance test

At the end of the treatment schedule, mice were given intraperitoneal injections of insulin (1 U/kg body weight). Blood glucose levels were determined by the tail-clip method at different time points as described previously [9].

2.5. Gel electrophoresis and western blotting

Liver tissues lysates were subjected to Western blot analysis as described previously [9] using phospho specific antibodies against Akt. Blots were then stripped and re-probed with antibodies directed against antibodies directed at the total protein.
2.6. Determination of total serum lipid

Serum levels of total cholesterol, high-density-lipoprotein (HDL) and triglycerides were measured using a commercial kits (from Equal Diagnostics, Exton, PA) and SpectraMax 340PC384 Microplate Reader System (Molecular Device, Sunnyvale, CA).

2.7. Measurement of lipid-peroxidation

The end product of lipid-peroxidation, namely malondialdehyde (MDA), was estimated in the liver tissue homogenates by the colorimetric kit (Bioxytech LPO-586, Portland, OR) using 1,1,3,3-tetra-ethoxypropane as standard according to the manufacturer’s specifications. This assay is based on the reaction of a chromogenic reagent N-methyl-2-phenylindole with MDA at 45 °C which yields a stable chromophore with absorbance maxima at 586 nm. Ability of Cr(D-Phe)₃ to inhibit in vitro lipid-peroxidation was assessed in rat-brain homogenates (10% w/v) by incubating Cr(D-Phe)₃ in the presence of hydrogen peroxide (1 mM) and ascorbic acid (1 mM) for 30 min at 37 °C followed by measuring MDA as described previously [13].

2.8. Quantification of protein-carbonyl

Protein-carbonyl content of total protein lysates from liver tissue was determined by colorimetrically estimating the product formed by the reaction of the carbonyls and 2,4-dinitrophenylhydrazine as described by us previously [14].

2.9. Data analysis

Data are expressed as mean ± SEM and statistically evaluated using Student’s paired t-test using Sigma Plot statistical software (Jandel Scientific, San Rafael, CA). A ‘p’ value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Effect of Cr(D-Phe)₃ ingestion on body mass

The first set of experiments were aimed at investigating whether ingestion of oral Cr(D-Phe)₃ at a dose of 150 µg/kg/day for a six-week period altered the overall body-mass in genetically obese, leptin-deficient C57BL/6J ob/ob (+/+ ) mice. The mean base-line total body weight and weights of heart, liver, and kidney for the ob/ob (+/+ ) mice and their age-matched lean counterparts are shown in Table 1. Compared to the lean animals, obese animals had significantly higher absolute weights of body, heart and liver as anticipated. Treatment of obese mice with Cr(D-Phe)₃ 150 µg/kg/day for a six-week period did not alter any of these body-mass indices.

![Graph](image)

Fig. 2. Effect of Cr(D-Phe)₃-treatment on insulin-sensitivity in obese mice. Following oral treatment with Cr(D-Phe)₃ (150 µg/kg/day for 6 weeks) mice were challenged with an intraperitoneal injection of insulin (1 U/kg body weight). Blood glucose levels were estimated prior to (0 min) and at various time following insulin challenge. Cr(D-Phe)₃-treated obese group (closed triangle) had a significantly lower glucose level compared to obese control animals (closed square). Closed circle indicated glucose level of lean control group. Values are means ± SEM, *p < 0.005, n = 12 versus vehicle-treated ob/ob (+/+ ) mice, at indicated time points.
the plasma glucose levels in both the obese and lean mice. However, in the chromium treated obese animals, the drop in glucose levels were significantly higher than that seen with untreated animals (115.3 ± 18.0 mg/dL versus 175.8 ± 43.2 mg/dL at 30 min post-challenge). In the lean mice, insulin-challenge failed to further lower the blood glucose level than that observed in the vehicle-treated group (figure not shown).

3.3. Effect of Cr(\textit{p}a)\textsubscript{3} on hepatic Akt-phosphorylation

Akt has been identified as an important kinase, downstream of insulin receptor necessary for insulin activity [15]. We have previously shown that Cr(\textit{d}-Phe)\textsubscript{3} can enhance the insulin-stimulated phosphorylation of Akt, in cultured adipocytes, suggesting that Akt may be a target protein for Cr(\textit{d}-Phe)\textsubscript{3} [9]. An increase in Akt phosphorylation was also observed in individuals with type-II diabetest who supplemented their diet with chromium picolinate had increased activity of Akt (in the skeletal muscles) compared to those who were on placebo (Cefalu et. al., 18th International Diabetes Federation Congress, 2003). Based on these reports we sought to investigate the effects of oral Cr(\textit{d}-Phe)\textsubscript{3}-treatment on Akt phosphorylation in the liver homogenate of \textit{ob/ob}(+/+) mice. Fig. 3 shows the levels of phospho-Akt in the liver-homogenates, as assessed by Western blotting using a phospho-specific Akt antibody. Phospho-Akt levels in obese mice were significantly lower that observed in lean animals, suggesting that Akt may have a role to play in obesity and type II diabetes. However, oral treatment with Cr(\textit{d}-Phe)\textsubscript{3} failed to enhance the levels of phospho-Akt in the obese mice. Neither did the treatment alter the levels of the total Akt protein.

3.4. Effect of Cr(\textit{d}-Phe)\textsubscript{3} on serum lipid profile

Recent reports have indicated that low-molecular weight organic chromium complexes can reduce fasting blood
plasma low-density lipoprotein cholesterol, total cholesterol and triglycerides in diabetic rats [16]. We therefore tested the ability of Cr(D-Phe)₃ to alter the lipid profile in obese animals. As shown in Fig. 4A, obese animals exhibited severe dyslipidemia compared to their lean counterparts as evidenced by significantly higher total serum cholesterol levels. Treatment with Cr(D-Phe)₃ caused a significant lowering of the total serum cholesterol levels (Fig. 4A). Serum levels of the beneficial HDL-cholesterol were higher in the obese animals compared to the lean mice (Fig. 4B). In contrast to serum cholesterol levels, treatment with Cr(D-Phe)₃ did not alter the levels of HDL-cholesterol. However, there was a significant decrease in the total serum cholesterol to HDL-cholesterol ratio in Cr(D-Phe)₃ animals: the ratios for the Cr(D-Phe)₃-treated and vehicle treated animals being 1.63 ± 0.05 and 2.19 ± 0.08, respectively. The difference in this ratio is thus attributable to the attenuation in serum cholesterol levels rather than any changes in the serum HDL levels. Similar to serum cholesterol and serum HDL, the serum triglycerides levels were higher in the obese animals compared to the lean controls (Fig. 4C). However, in contrast to its effects on serum cholesterol, Cr(D-Phe)₃-treatment caused a further increase in serum triglycerides (Fig. 4C).

3.5. Effect of Cr(D-Phe)₃ on oxidant stress

Oxidant stress has been implicated as one of the causes and also an important consequence of diabetes and lipid dysfunctions [17]. The levels of oxidant stress in liver homogenates as evidenced by the extent of lipid-peroxidation and protein carbonyl formation were significantly higher in obese, diabetic animals compared to lean animals. Cr(D-Phe)₃-treated animals had lower levels of these oxidative-stress markers compared to the untreated obese animals (Fig. 5A and B). Besides in vivo lipid-peroxidation, Cr(D-Phe)₃ also inhibited in vitro lipid-peroxidation stimulated by hydrogen peroxide and ascorbate in rat-brain homogenates in a concentration-dependent manner (Fig. 6). These results suggest that the complex may possess a direct inhibitory effect on lipid-peroxidation.

4. Discussion

We have recently reported that Cr(D-Phe)₃, a novel chromium complex, enhances insulin signaling and glucose-uptake in cultured adipocytes [9]. The present studies were designed to investigate the effects of this complex on insulin-sensitivity and plasma lipid profile in vivo. Here, we show that treatment of genetically-obese, leptin-deficient mice with Cr(D-Phe)₃ improves insulin-sensitivity and lowers total serum cholesterol. These results suggest that Cr(D-Phe)₃ may have beneficial effects in the prophylaxis and treatment of type II diabetes and obesity.
In addition to its effects on glucose, insulin, and lipid metabolism, chromium has been reported to increase lean body mass and decrease percentage body fat, which may lead to weight loss in humans [18]. In our experiments however, Cr(D-Phe)₃ did not alter the total body weight of obese animals indicating that the improvement in carbohydrate and lipid-metabolism did not translate into reduction of obesity in the model tested. One possible explanation for this may be the short-term treatment schedule used in these experiments. Further experiments with chronic ingestion of Cr(D-Phe)₃ is warranted to ascertain whether this compound can be of use in the treatment of obesity.

Our previous studies suggest that the insulin-stimulatory effect of Cr(D-Phe)₃ may be attributed to the ability of the complex to augment insulin-stimulated phosphorylation of key proteins such as the insulin-receptor beta and Akt in the insulin-signal-transduction cascade [9]. A recently concluded clinical study suggests that chromium picolinate enhances the phosphorylation of Akt in skeletal muscles of diabetic patients (Cefalu et al., 18th International Diabetes Federation Congress, 2003). In our in vivo studies however, treatment with Cr(D-Phe)₃ failed to enhance the levels of phospho-Akt in obese mice. Neither did we observe any change in the phosphorylation of insulin-receptors on reprobing the same blots against antibodies directed against the phosphorylated insulin receptor beta (figure not shown). One possible explanation for this lack of effect may be the fact that we are looking at the basal phosphorylation levels as against insulin-stimulated phosphorylation, which was the case with our in vitro experiments.

Several previous studies in experimental animals and human subjects have shown that chromium therapy may have beneficial effects in hyperlipidemic conditions [4,16,19]. The results of the present study support this notion: Cr(D-Phe)₃ treatment lowered total serum cholesterol without altering HDL-cholesterol. Leptin-deficient ob/ob mice used in this study may not be an ideal model to study HDL owing to the fact that the basal HDL-cholesterol levels were significantly higher in these animals compared to the lean controls (Fig. 4, panel B). In contrast to its effects on plasma cholesterol, treatment of obese mice with Cr(D-Phe)₃ showed an increase in serum triglycerides which contradicts some previous observations suggesting that chromium complexes can lower serum triglyceride levels [16,19].

The picolinate ligand in the most popularly used chromium supplement, chromium picolinate, has been recently reported to shift the redox potential of chromium in the complex such that it can be reduced by biological reductants to generate hydroxyl radicals causing deleterious DNA mutations [8,20]. In contrast to these findings, studies by Jain et al. [21] have shown that chromium chloride can inhibit oxidative stress induced by high glucose and hydrogen peroxide in cultured monocytes. Thus, the toxicity of chromium complex has been attributed to chromium picolinate but not chromium chloride indicating that the safety of chromium(III) is largely dependent on the ligand to which it is complexed [22]. Previous studies suggest that chromium complexed with amino acid ligands do not cause DNA nicking [9,11]. The data presented here shows that Cr(D-Phe)₃ attenuates oxidant stress in vivo and in vitro. Thus, not only are chromium–amino acid complexes devoid of the DNA-damaging effects attributed to chromium picolinate, these complexes may also possess a direct antioxidant property. There are several reports that link the pathophysiology of diabetes and lipid-disorders to oxidant stress [17,23]. The ability of Cr(D-Phe)₃ to lower oxidative stress may thus add to its therapeutic value in treating diabetes and comorbid conditions.

In summary, in this report we demonstrate that oral administration of Cr(D-Phe)₃ improves insulin-sensitivity, reduces total plasma cholesterol levels and attenuates hepatic oxidant stress in a mice model of type-II diabetes and obesity. Taken together, these data suggest that Cr(D-Phe)₃ may be of potential value in the therapy or prophylaxis of insulin-resistance and dyslipidemia associated with obesity.

5. Abbreviations

Cr(D-Phe)₃ chromium (III) (D-phenylalanine)
LMW low molecular weight
HDL high-density lipoprotein
MDA malondialdehyde

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