A newly synthetic chromium complex—Chromium (p-phenylalanine)$_3$ activates AMP-activated protein kinase and stimulates glucose transport

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1. Introduction

Insulin resistance is concomitant with type 2 diabetes, obesity, hypertension, and metabolic syndrome. Chromium, an essential transition metal, may be an important addition to type 2 diabetes treatment choices because it is thought to play a role in glucose metabolism by potentiating insulin action [1]. Animal studies have shown that a deficiency in dietary...
chromium can result in an inability to remove glucose efficiently from bloodstream [2]. Clinical trials have demonstrated that supplementation with chromium chloride or chromium picolinate can lower blood glucose levels in diabetic patients [3]. Emerging evidence has shown that the biological active form of chromium is a chromium-oligopeptide complex, which justifies the use of organic chromium-complexes as biomimetic chromium supplements [4]. To increase the bioavailability of chromium, we synthesized a new complex of chromium with phenylalanine, chromium (³-phenylalanine)₃ [Cr(³-phe)₃] [1,5], and found that Cr(³-phe)₃ can improve insulin responsiveness and reduce whole body glucose tolerance of mice [1,6,7].

The exact mechanism whereby Cr(³-phe)₃ participates in the functions of insulin has not been elucidated, even though Cr(³-phe)₃ potentiates insulin-stimulated Akt (Thr³⁰⁸) phosphorylation in 3T3-adipocytes [1]. There is more evidence that AMP-activated protein kinase (AMPK) is a serine-threonine kinase which has an important role in the regulation of cellular metabolism [8,9], ion channels [10] and gene expression [11]. AMPK is activated by increases in the AMP:ATP ratio, and as such, is a key signaling pathway during cellular metabolic or energetic stress. AMPK was initially found to be an important regulator of fatty acid oxidation in heart [12,13] and skeletal muscle [14], but has also emerged as an important mediator of glucose metabolism [15]. AMPK also activates phosphofructokinase-2, which accelerates glycolysis [16].

The downstream mechanisms through which AMPK mediates the acute activation of glucose transport remain uncertain. However, AMPK may potentially mediate its effect on glucose transport in part through interaction with the nitric oxide pathway. AMPK phosphorylates endothelial nitric oxide synthase (eNOS) on Ser¹¹⁷⁷ [17–19], leading to NOS activation in a calcium-independent fashion. However, the extent to which NOS modulates glucose uptake and glucose transporter (GLUT) translocation in heart muscle remains uncertain. In skeletal muscle from eNOS knockout mice, there is diminished insulin-stimulated glucose uptake, indicating that insulin activation of nitric oxide may contribute to the stimulation of glucose transport [20–22].

The purpose of this study was to determine whether AMPK-activation is involved in Cr(³-phe)₃-stimulated glucose uptake and to investigate the potential mechanisms by which Cr(³-phe)₃ triggers AMPK signaling pathways in myocardial cells and cardiomyocytes. The results indicate that Cr(³-phe)₃ stimulates Akt and AMPK signaling pathways, both of which contribute to Cr(³-phe)₃ triggered glucose transport in H9c2 myoblast cells. Moreover, the energy stress, i.e. the increasing cytosolic AMP concentration and decreasing mitochondrial membrane potential (Δψₘ), caused by Cr(³-phe)₃ may be the stimulus for the activation of AMPK signaling pathways.

2. Materials and methods

2.1. Structure of Cr(³-phe)₃

The synthesis and characterization of Cr(³-phe)₃ were as described previously [1]. The structure of Cr(³-phe)₃ as follow:

![Structure of Cr(D-phe)₃](image)

2.2. Cell line and animals

H9c2 cells, a clonal cell line derived from fetal rat heart, purchased from American Type Culture Collection (ATCC, Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY) and 1% penicillin and streptomycin and maintained in 95% air and 5% CO₂ at 37 °C. Cells were passaged regularly and subcultured to 90% confluence before experiments. 10–12 weeks age of male FVB mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal procedures used in this study were approved by the Animal Care and Use Committees at the University of Wyoming. All animals were kept in our institutional animal facility at the University of Wyoming with free access to standard laboratory chow and tap water.

2.3. Isolation of mouse cardiomyocytes

Cardiomyocytes were enzymatically isolated as described previously [23]. In brief, hearts were removed and perfused with oxygenated (5% CO₂/95% O₂) Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM) 118 NaCl, 4.7 KCl, 1.25 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 10 HEPES and 11.1 glucose. All the chemicals were purchased from Sigma (St. Louis, MO). Heats were then perfused with a Ca²⁺-free KHB containing Liberase Blendzyme 4 (Hoffmann-La Roche Inc., Indianapolis, IN) for 20 min. After perfusion, left ventricles were removed and minced to disperse cardiomyocytes. Extracellular Ca²⁺ was added incrementally back to 1.25 mM. The myocytes were treated with Cr(³-phe)₃ for AMPK signaling, glucose uptake and mitochondrial membrane potential measurements.

2.4. JC-1 assay

5,5',6,6'-Tetrachloro-1,1',3,3'-tetraethylbenzimidazolocarboxylic acid iodide (JC-1, Sigma, St. Louis, MO) is a positively charged fluorescent compound which is taken up by mitochondria proportionally to the inner mitochondrial membrane...
potential [24]. When a critical concentration is reached, JC-1 monomer forms J-aggregates, altering the fluorescence properties of the compound. Thus, the ratio of red (J-aggregate)/green (monomeric JC-1) emission is directly proportional to the mitochondrial membrane potential (ΔΨ). Isolated cardiomyocytes were suspended in HEPES-saline buffer and pre-incubation with 10 μM JC-1 for 10 min at 37 °C. Fluorescence of each sample was read at excitation wavelength of 490 nm and emission wavelength of 530 nm and 590 nm using a spectrofluorimeter. Results in fluorescence intensity were expressed as 590-to-530-nm emission ratio [25].

2.5. Immunoblotting

Immunoblots were performed as previously described [26]. The cell or heart homogenate proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). For reprobing, membranes were stripped with 50 mM Tris–HCl, 2% SDS, and 0.1 M β-mercaptoethanol (pH 6.8) (Bio-Rad, Hercules, CA). Rabbit polyclonal antibodies against phospho-AMPK total AMPKα, phosphor-eNOS and GAPDH were purchased from Cell Signaling (Danvers, MA). Rabbit polyclonal antibodies against phospho-acetyl-CoA carboxylase (p-ACC) and total ACC were from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies against phosphor-eNOS and GAPDH were purchased from Cell Signaling (Danvers, MA). Rabbit polyclonal antibodies against phospho-acetyl-CoA carboxylase (p-ACC) and total ACC were from Upstate Biotechnology (Lake Placid, NY). The intensity of bands was measured with a scanning densitometer (model GS-800; Bio-Rad) coupled with Bio-Rad personal computer analysis software.

2.6. Glucose uptake

2-Deoxy-d-[1-3H]glucose accumulation in H9c2 cells or cardiomyocytes was performed as previously described [1,27]. H9c2 cells grown in 6-well plates were washed twice with serum-free DMEM and incubated with 3 ml of the medium at 37 °C for 2 h. The cells were washed 3 times with Krebs-Ringer-HEPES (KRH) buffer and incubated with 3 ml KRH buffer at 37 °C for 30 min. Insulin (10 nM, Sigma, St. Louis, MO) or Cr(α-phe)3 (25 μM) and/or Compound C (20 μM, Calbiochem, Gibbstown, NJ) or Wortmannin (0.5 mM, Calbiochem, Gibbstown, NJ) were then added to H9c2. Glucose uptake was initiated by the addition of 0.1 ml KRH buffer and 2-deoxy-d-[1-3H]glucose (0.2 1 Ci/ml, GE Healthcare, Piscataway, NJ) and 5 mM glucose as final concentrations. Glucose uptake was terminated by washing the cells three times with cold PBS. The cells were lysed overnight with 1 ml 0.5 M NaOH and 0.1% SDS (w/v). The radioactivity retained by the cell lysates was measured by a scintillation counter (Beckmann LC 6000IC) and normalized to protein amount measured with a Micro BCA Protein Assay Kit (Pierce Chemical, Rockford, IL).

2.7. Measurement of nucleotides by HPLC

The heart muscles were extracted by 6% perchloric acid (Sigma, St. Louis, MO) [28]. The acidic homogenate was kept on ice for 30 min, and then centrifuged at 14,000 rpm at 4 °C for 10 min. An aliquot of the pellets was set aside for protein measurements. The supernatant was neutralized with 1 M K2CO3, adjust pH to 3.5. Then kept the supernatant on ice for 10 min and at −80 °C for 1–2 h to promote precipitation of the perchlorate and centrifuged again. Supernatants were stored at −80 °C until HPLC assay. The chromatographic separation of AMP was performed using a Grace Partisil SAX column (250 mm × 4 mm i.d., particle size 10 μm) (Deerfield, IL). The mobile phases were composed of a gradient of 5 mM ammonium dihydrogen phosphate (Sigma, St.Louis, MO, pH 2.8) and 750 mM ammonium dihydrogen phosphate (pH 3.9). The flow rate was varied from 1 to 2 ml min−1 over the course of the gradient profile to provide a reasonable assay time of 25 min. The sample injection volume was 50 μl and the components were monitored at 254 nm. The Beckman GOLD HPLC system was operated in laboratory at room temperature (23–25 °C). Concentrations were determined by construction of a calibration curve range from 1 to 80 nmol per 50 μl injected. Standard stock solutions for calibration curve construction were 6.4 μmol ml−1 AMP and ATP prepared in 5 mM ammonium dihydrogen phosphate (pH 2.8). These solutions were stored at −80 °C and used as references for peaks quantification. Fresh dilution was made before each assay to construct a calibration curve, adding 5 mM ammonium dihydrogen phosphate (pH 2.8) in order to obtain 1, 5, 10, 20, 40 and 80 nmol per 50 μl injected.

2.8. Statistical analysis

For each experimental series, data are presented as mean ± SE. Statistical significance (P < 0.05) for each variable was estimated by analysis of variance (ANOVA) or t-test, where appropriate.

3. Results

3.1. Chromium complex-Cr(α-phe)3 activates AMPK signaling pathways in H9c2 myoblast cells and cardiomyocytes

AMPK is not only an important mediator of glucose transporter in the heart during ischemia [29], but also a regulator in increasing insulin sensitivity [30]. To determine whether AMPK signaling pathway is involved in Cr(α-phe)3 stimulation of glucose transport, we treated H9c2 myoblast cells or cardiomyocytes with Cr(α-phe)3. The results demonstrated that Cr(α-phe)3 induced the phosphorylation of α catalytic subunit at Thr172 of AMPK in H9c2 cells (Fig. 1A) and in cardiomyocytes (Fig. 3A), as well as the phosphorylation of downstream of AMPK, i.e. acetyl-CoA carboxylase (ACC) and eNOS in H9c2 cells (Fig. 1B).

3.2. AMPK signaling pathway mediates Cr(α-phe)3 stimulated glucose uptake

AMPK is not only a key regulator in glucose transport, but also a regulator in increasing insulin sensitivity [30]. To determine whether AMPK signaling pathway is involved in Cr(α-phe)3 stimulation of glucose transport, we treated H9c2 myoblast cells or cardiomyocytes with Cr(α-phe)3. The results demonstrated that Cr(α-phe)3 induced the phosphorylation of α catalytic subunit at Thr172 of AMPK in H9c2 cells (Fig. 1A) and in cardiomyocytes (Fig. 3A), as well as the phosphorylation of downstream of AMPK, i.e. acetyl-CoA carboxylase (ACC) and eNOS in H9c2 cells (Fig. 1B).

3.2. AMPK signaling pathway mediates Cr(α-phe)3 stimulated glucose uptake

We have reported that Cr(α-phe)3 improved insulin-stimulated glucose uptake in cultured 3T3-adipocytes [1]. The effect of Cr(α-phe)3 on glucose uptake in H9c2 myoblast cells and cardiomyocytes was investigated using the 2-deoxy-d-[1-3H]glucose uptake assay [29]. As shown in Figs. 2 and 3B, Cr(α-phe)3 (25 μM) significantly stimulated glucose uptake in H9c2 cells (Fig. 2) and in cardiomyocytes (Fig. 3B). P3 kinase inhibitor, Wortmannin or AMPK inhibitor, Compound C
AMP-activated protein kinase (AMPK) and Akt signaling pathway activation of H9c2 myoblast cells stimulated by chromium ( β-phenylalanine) [Cr( β-phe)]. Representative immunoblots of H9c2 cell lysate (A) phosphorylated (Thr$^{172}$) AMPK (p-AMPK) or pan-α-subunit (AMPKα). Phosphorylated AMPK was quantified relative to total amount of AMPKα. Values are means ± SE. (n = 4) * P < 0.05 vs. control. (B) phosphorylated (Ser$^{473}$) Akt (p-Akt) or total Akt (Akt). Phosphorylated Akt was quantified relative to total amount of Akt. Values are means ± SE. (n = 4) * P < 0.05 vs. control. (C) phosphorylated AMPK downstreams, ACC (Ser$^{212}$) and eNOS (Ser$^{1177}$). Phosphorylated ACC and eNOS were quantified relative to the amount of loading control β-Tubulin. Values are means ± SE. (n = 5), * P < 0.01 vs. control.
partially inhibited the Cr(D-phe)₃ stimulation of glucose uptake (Figs. 2 and 3B), while Wortmannin plus Compound C abrogated the glucose uptake induced by Cr(D-phe)₃ (Figs. 2 and 3B). While Compound C did not affect the insulin stimulation of glucose uptake in H⁹c² cells, Wortmannin pretreatment abolished glucose uptake triggered by insulin (Fig. 2). These results indicate that both Akt and AMPK signaling pathways play a role in mediating Cr(D-phe)₃ stimulation of glucose transport in H⁹c² myoblast cells.

3.3. Cr(D-phe)₃ activates cardiac AMPK signaling pathway in vivo

We first utilized an in vivo model in order to examine whether Cr(D-phe)₃ activates AMPK in the intact heart. Intraperitoneal (i.p.) injection of Cr(D-phe)₃ (1 mg/kg) in anesthetized FVB mice stimulated cardiac AMPK activation (Fig. 4A) and downstream ACC phosphorylation (Fig. 4B). This result demonstrates that Cr(D-phe)₃ activates cardiac AMPK signaling pathway in vivo, which suggest that Cr(D-phe)₃ may have a cardioprotective effect to prevent ischemic injury via activation of AMPK signaling [31].

3.4. Cr(D-phe)₃ reduces the mitochondrial membrane potential of cardiomyocytes

To understand the mechanisms of Cr(D-phe)₃ activation of cardiac AMPK signaling pathway, the mitochondrial membrane potential was assessed using the dye JC-1, a lipophilic fluorophore that forms J-aggregates in proportion to its intramitochondrial concentration, dictated by the mitochondrial membrane potential (ΔΨ). Isolated cardiomyocytes were preincubated for 20 min with 10 μM JC-1, rinsed thoroughly and treated with 25 μM Cr(D-phe)₃, before addition of 2-deoxy-[1-³H]glucose for 30 min to measure glucose uptake. For inhibitors experiments, cardiomyocytes were preincubated for 15 min with Wortmannin (0.5 mM) or/and Compound C (10 μM) before incubation with Cr(D-phe)₃ (25 μM) for 30 min to determine the effect on glucose uptake. Values are means ± SE for 4 experiments. *P < 0.05 vs. control groups, respectively, †P < 0.05 vs. Cr(D-phe)₃ alone.

Fig. 2 – Stimulation of glucose uptake by Cr(D-phe)₃. H⁹c² myoblast cells were preincubated for 30 min with or without 25 μM Cr(D-phe)₃, with insulin (10 nM) for positive control, before addition of 2-deoxy-[1-³H]glucose for additional 30 min to measure glucose uptake. For inhibitors experiments, H⁹c² cells were preincubated for 15 min with Wortmannin (0.5 mM) or/and Compound C (10 μM) before incubation with or without Cr(D-phe)₃ (25 μM)/insulin (10 nM) for 30 min to determine the effect on glucose uptake. Values are means ± SE for 5 experiments. *P < 0.05 vs. control groups, respectively, †P < 0.05 vs. insulin alone, ‡P < 0.05 vs. Cr(D-phe)₃ alone.

Fig. 3 – Stimulation of AMPK signaling pathway and glucose uptake by Cr(D-phe)₃ in cardiomyocytes. (A) Representative immunoblots of phosphorylated (Thr172) AMPK (p-AMPK), pan-α-subunit (AMPKa) or β-Tubulin. Phosphorylated AMPK was quantified relative to total amount of AMPKa. Values are means ± SE. (n = 5) *P < 0.05 vs. control. (B) Isolated cardiomyocytes were preincubated for 30 min with or without 25 μM Cr(D-phe)₃, before addition of 2-deoxy-[1-³H]glucose for additional 30 min to measure glucose uptake. For inhibitors experiments, cardiomyocytes were preincubated for 15 min with Wortmannin (0.5 mM) or/and Compound C (10 μM) before incubation with or without Cr(D-phe)₃ (25 μM) for 30 min to determine the effect on glucose uptake. Values are means ± SE for 4 experiments. *P < 0.05 vs. control groups, respectively, †P < 0.05 vs. Cr(D-phe)₃ alone.
15 min) did not cause mitochondrial membrane depolarization (data not shown). Cr(D-phe)3 activates cardiac AMPK might be associated with reduction of mitochondrial membrane potential (Δψ).

3.5. Cr(D-phe)3 treatment causes increase of cytosolic concentration of AMP

As shown in Fig. 4, Cr(D-phe)3 activates cardiac AMPK and induces phosphorylation of downstream ACC in the intact hearts. In Cr(D-phe)3-treated hearts, cytosolic [AMP] was measured by HPLC [32]. The results demonstrated that Cr(D-phe)3 caused increase of [AMP] in a time dependent manner (Fig. 5B), suggesting that Cr(D-phe)3 induced activation of the cardiac AMPK signaling pathway may be mediated by reduction of mitochondrial membrane potential (Δψ) and increase the cytosolic AMP, which can allosterically activate AMPK activity through binding the γ subunit of AMPK [33].

3.6. Discussion

Chromium, an essential transition metal suggested as being beneficial in individuals with glucose intolerance, type 2 diabetes, gestational diabetes and steroid-induced diabetes, shows evidence of enhancing insulin action and glucose transport at the molecular level [4,34,35]. Our results demonstrated that Cr(D-phe)3 can stimulate cardiac AMPK signaling pathways in vitro and in vivo. Moreover, Cr(D-phe)3 significantly stimulated glucose uptake in both H9c2 cells and isolated cardiomyocytes. AMPK inhibitor Compound C markedly inhibited the glucose uptake stimulated by Cr(D-phe)3, while it did not affect insulin stimulation of glucose uptake in H9c2 cells, which indicated that AMPK play a role in modulating Cr(D-phe)3-stimulated glucose transport. The increase of cardiac AMP concentration and decrease of mitochondrial membrane potential (Δψ) may contribute to the activation of AMPK induced by Cr(D-phe)3.

Chromium deficiency has been associated with hyperglycemia in test animals as well as humans which can be reversed by chromium supplementation [36–38]. Moreover, chromium has also been shown to have a beneficial effect on individuals with no diabetic symptoms. Serum chromium levels in healthy individuals were found to be inversely related to insulin peaks in response to a glucose challenge [3,39]. The observed rapid decrease in chromium in response to glucose was not due to urinary excretion [39]. In diabetic volunteers chromium levels were not found to fluctuate with respect to insulin [39]. Hypoglycemia is on the opposite end of the glucose/insulin axis. If chromium is a key factor in overall glucose/insulin homeostasis, it should be effective in controlling clinical symptoms of low blood sugar. Treatment of type 2 diabetes with chromium has led to improvement in blood glucose, insulin, and hemoglobin A1C levels [40]. The use of organic chromium complexes has been found to give superior results when compared to inorganic salts [41].

Accumulated evidences have showed that chromium complexes such as chromium picolinate and chromium-based energy formula can enhance insulin-stimulated glucose transport and protect cardiomyocytes from ischemia-reperfusion injury [34,46]. To our knowledge, this is the first study that examined the role of this new chromium complex-chromium (phenylalanine)3 [Cr(D-phe)3] in regulation of cardiac glucose transport. The increases glucose transport by Cr(D-phe)3 as shown here, may at least in part provide novel molecular
insight by which Cr(D-phe)₃ supplementation might be beneficial for individuals with impaired glucose and lipid metabolism.

Macrovascular disease develops in parallel with the development of type 2 diabetes mellitus [42], however, it is sometimes present before the clinical onset of diabetes mellitus. Numerous studies suggest that an increase in postprandial plasma glucose levels in non-diabetic patients is a strong predictor of cardiovascular events [43,44]. AMPK has been described as the ‘energy sensor’ or ‘gauge’ of a cell since its activity is altered by the cellular energy state, and it regulates a number of metabolic processes to restore energy depletion in both the periphery and central nervous system [30]. We have found that AMPK plays an important role in regulation of glucose transport in the heart during ischemic stress and AMPK activation promotes glucose uptake, glycolysis and limits apoptosis and cell damage [27,29,31,45]. Interestingly, chromium has been shown to enhance myocardial protection from ischemia/reperfusion injury as well [46]. However, none of the studies have checked that whether Cr(D-phe)₃ can activate of cardiac AMPK and enhance glucose transport in cardiomyocytes. Therefore, based on our results, Cr(D-phe)₃ could have strong potential for development of cardioprotective drugs in the future as a new AMPK activator.

There are two known pathways for AMPK regulation in mammalian cells. The first and best defined pathway involves increased AMP binding to the cystathionine β-synthase (CBS) domains of AMPK-γ [26]. This increases Thr₁₇₂ phosphorylation of a subunit of AMPK by upstream AMPK kinases (AMPKK), which is required for increased AMPK activity [47]. The second pathway for AMPK activity regulation is directed by Ca²⁺/calmodulin-dependent protein kinase kinase (CaMKK) activity [48]. AMP binding to AMPK-γ is not required, CaMKK activity increases in response to increased Ca²⁺ [49]. This pathway for AMPK regulation by CaMKK, which is dependent on intracellular Ca²⁺, corresponds to a metabolic regulation function for AMPK at the organism level [30]. Our results indicate that one of mechanisms by which Cr(D-phe)₃ activates cardiac AMPK signaling pathway may mediate increase [AMP] via reduction of mitochondrial membrane potential (Fig. 5). The activation of AMPK may activate ATP-generating pathways, like glycolysis [30], so after Cr(D-phe)₃ treatment for 60 min, the [AMP] appears to be reduced (Fig. 5B). There are emerging evidences that chromium could cause the change of intracellular redox status [50], which may impact the mitochondrial membrane potential [51]. It could result in disturbance of the energy balance, which lead to increase concentrations of AMP, trigger of AMPK signaling pathway [45].

AMPK is now widely accepted as a therapeutic target for the treatment of type 2 diabetes, obesity and anorexia based on its actions on feeding and whole body metabolism [52]. Most research to date has been aimed at the treatment of type 2 diabetes that affects ~162 million people worldwide. There is abundant evidence that AMPK influences glucose and lipid metabolism including the observations that activation of AMPK increases glucose transporter GLUT4 translocation in skeletal muscles [53]. There may also be a therapeutic role for AMPK in the treatment of stroke and neurodegeneration since activation of AMPK by AMPK activator AICAR confers protection to hippocampal neurons following glucose deprivation, glutamate, excitotoxicity, hypoxia and Aβ peptide [54].

Taken together, dietary chromium supplements are inexpensive, and the limited safety data suggest that chromium is safe even at high doses [41]. Cr(D-phe)₃ as a novel AMPK activator would be an attractive option for management of diabetes and ischemic heart diseases, and for the control of insulin and glucose concentrations of persons at high risk of type 2 diabetes.

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