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Chromium Improves Glucose Uptake and Metabolism Through Upregulating the mRNA Levels of IR, GLUT4, GS, and UCP3 in Skeletal Muscle Cells

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Abstract The aim of this study was to evaluate the impact of three different chromium forms as chromic chloride (CrCl), chromium picolinate (CrPic), and a newly synthesized complex of chromium chelated with small peptides (CrSP) on glucose uptake and metabolism in vitro. In cultured skeletal muscle cells, chromium augmented insulin-stimulated glucose uptake and metabolism as assessed by a reduced glucose concentration of culture medium. At the molecular level, insulin significantly increased the mRNA levels of insulin receptor (IR), glucose transporter 4 (GLUT4), glycogen synthase (GS), and uncoupling protein-3 (UCP3), and these impacts can be enhanced by the addition of chromium, especially in the form of CrSP. Collectively, results of this study demonstrate that chromium improves glucose uptake and metabolism through upregulating the mRNA levels of IR, GLUT4, GS, and UCP3 in skeletal muscle cells, and CrSP has higher efficacy on glucose uptake and metabolism compared to the forms of CrCl and CrPic.

Keywords Chromium · Insulin · Glucose uptake · Glucose metabolism · Insulin receptor · Glucose transporter 4 · Glycogen synthase · Uncoupling protein-3

All authors listed have contributed to the work and have agreed to submit the manuscript to Biological Trace Element Research. The manuscript is original, has not been published before, and is not under consideration for publication elsewhere.

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Introduction

Chromium (Cr) is an essential nutrient of humans and animals. Despite over five decades of endeavor, the role of Cr at a molecular level has been a poorly understood field of study. Several investigations with animals and humans provide evidence that the essential element chromium is required to achieve optimal action of insulin [1]. Inorganic forms, such as that present in chromic chloride ($CrCl_3$; as hexahydrate) and chromic oxide (Cr_2O_3), are absorbed poorly; organic chromium complexes, such as chromium picolinate (CrPic), appeared to have better effects. The picolinate form has been reported to be the best absorbed and most efficacious. However, concerns have arisen over its use, especially with regards to potential clastogenic damage [2, 3].

There is a need to find novel Cr compounds. Recently, it was reported that chromium chelated with amino acid appears to be the most effective [4, 5]. More recent studies have suggested that chromium may function as part of the oligopeptide low-molecular-weight chromium (LMWCr)-binding substance (MW 1,500 Da) [1]. In our previous studies, we demonstrated that the absorptivity of CrSP was greater than that of CrPic [6]. We want to know if CrSP has better bioactivity.

Chromium has been shown to improve insulin-stimulated glucose uptake in cultured cells sensitive to insulin [7]. Chromium is thought to increase insulin binding to cells, to enhance the insulin receptor number, and to potentiate insulin receptor kinase activity [8]. Most of the previous studies concentrated upon the direct effects or factors of insulin, and the exact mode of action of chromium is unknown. glycogen synthase (GS) regulates glycogen synthesis and plays a role in the storage of glucose. UCP3 stimulates glucose transport and GLUT4 translocation to the cell surface in skeletal muscle cells by activating a phosphoinositide 3-kinase-dependent pathway [9]. Fewer reports exist about the effect of chromium on the gene expression regulation of IR and GLUT4. However, the effects of chromium supplementation on the gene expression regulation of GS and UCP3 still remain unclear.

The objective of the study was to determine the effect of various forms of chromium on glucose uptake and metabolism in the absence or presence of insulin. Skeletal muscle was chosen as an experimental model, as this tissue accounts for more than 80% of glucose disposal in the body. Hence, we examined the effect of various chromium compounds on glucose uptake and metabolism and gene expression in cultured skeletal muscle cells.

Materials and Methods

Materials

Rat L6 myoblasts were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), penicillin–streptomycin liquid, and fetal calf serum (FCS), were purchased from Gibco (Burlington, Ontario, Canada). Insulin was obtained from Sigma (St. Louis, MO, USA). Doubly deionized water was used to prepare stock solutions of chromium complex for all experiments unless otherwise indicated.

Chromium chloride and chromium picolinate were kind gifts from Dr. Zhang (Mianyang Sinyiml Chemical Co., Ltd, CN). CrSP was provided by the Engineering Research Center of Animal Disease-Resistance Nutrition, Ministry of Education, China. CrSP is a patented mixture of small peptides (Chinese patent no. CN1907086A). The small peptides were

hydrolysate of proteins. Analysis results of hydrolysate by high-performance liquid chromatography indicated that more than 90% of its compositions were dipeptides and tripeptides. The mole ratio of Cr/peptide is 1:3.

Cell Culture

Rat L6 skeletal muscle myoblasts were initially established for 48 h in DMEM supplemented with 10% FCS and antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin) in an incubator under a humidified atmosphere containing 5% CO_2 and 95% air at 37°C. Thereafter, the cells were cultured in serum-free treatment medium which was replaced daily. Each of the culture treatment media contained Dulbecco's modified Eagle's medium, with or without 100 nM insulin. One medium contained 0.5 μM chromium chloride, a second contained 0.5 μM chromium picolinate, a third contained 0.5 μM CrSP, and the fourth medium contained no chromium. After 72 h, the culture medium was collected for glucose uptake analysis, and the cells were harvested and used for gene expression analysis.

Glucose Uptake Assay

Glucose concentration of the culture medium after 72 h (designated as *B*, mM) was determined by commercial kit (Jiancheng Biochemical Reagent Co., Nanjing, China). The original glucose concentration of the culture medium was 25 mM (designated as *A*). The ΔC of glucose=$A-B$. Then, the value of C could be regarded as the glucose which had been metabolized by the muscle cells.

Analysis of IR, GLUT4, GS, and UCP3 mRNA Levels by Real-Time PCR

In the experiment, the total RNA was isolated from the skeletal muscle cell samples using TRIzol reagent (TaKaRa, Shiga, Japan) according to the manufacturer's protocol. Aliquots of the RNA samples were used for real-time polymerase chain reaction (PCR). Reverse transcription (RT) was performed with total RNA pretreated with DNase I (Fermentas) to obtain cDNA with SuperScript II and Oligo(dT) 18 (Invitrogen) as the primer in a 20-μL reaction volume. Each cDNA sample was diluted 1:4 in sterile ddH_2O, and 1 μL of this dilution was used as template for quantitative PCR. The cDNAs were stored at −20°C for analysis of IR, GLUT4, GS, and UCP3 mRNA levels by real-time PCR. β-actin was used as the housekeeping gene [10]. Sequences of primers used for quantitative real-time PCR analysis are shown in Table 1. Primers for the PCR reactions were designed by DNAStar (DNASTAR Inc.) to have a T_m of 62–65°C and an optimal annealing temperature of 63°C with the length of the amplicons between 100 and 250 bp. Real-time PCR was performed with SYBR® Premix Ex Taq™ (TaKaRa) in 20-μL reactions using the iCycler System (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instruction. Each PCR reaction contains 1 μL of cDNA, 0.2 μM of each of the primers, and 10 μL of master mix. The initial denaturing time was 1 min at 95°C, followed by 40 cycles consisting of 94°C for 15 s, 63°C for 20 s, and 72°C for 30 s with a single fluorescence measurement. A melting curve (65°C–95°C with a heating rate of 0.05°C s^{-1} and a continuous fluorescence measurement) was run after the PCR cycles followed by a cooling step at 40°C. For relative quantification, amplification efficiencies (Eff.) for each gene were determined as follows: a portion of cDNAs transcribed from 5 μg of total root RNA was diluted with sterile ddH_2O to be 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}. Standard curves for each gene were performed using the original and diluted cDNAs covering the range of all template

Table 1 Sequences of Primers Used for Quantitative Real-Time PCR Analysis

Target gene	Primer sequences		Product size (bp)	Genebank accession no.
IR	Forward	5′-GCTTTCGCGGGGTTGGGCTTTGA-3′	246	NM 017071
	Reverse	5′-CCCCCACCCCACCCTGTGAC-3′		
GLUT4	Forward	5′-CTGGCCCCATCCCCTGGTTCAT-3′	234	NM 012751
	Reverse	5′-CAAATGTCCGGCCTCTGGTTTCAG-3′		
GS	Forward	5′-GTCGGTCAACCCCGCAACTGCT-3′	227	XM 001077000
	Reverse	5′-AGGAGGGGCCTGCCCATTCACT-3′		
UCP3	Forward	5′-ACCTTTCCGGTTGGATGCCTAACAT-3′	236	NM 013167
	Reverse	5′-ATTACAGGGCGGAGTCATCTGGTGA-3′		
β-actin	Forward	5′-CTCCTCCCTGGAGAAGAGCTA -3′	248	NM 031144
	Reverse	5′-CCTTCTGCATCCTGTCGGCAA -3′		

IR insulin receptor, *GLUT4* glucose transporter 4, *GS* glycogen synthase, *UCP3* uncoupling protein-3

concentrations. Real-time PCR efficiencies (Eff.) were calculated from the given slopes in the iCycler software of the standard curves according to the equation $E=10^{-1}$/slope. Crossing points, defined as the point at which the fluorescence rises above the background fluorescence, was determined using the "fit point method" in the iCycler System (Bio-Rad Laboratories). Gene-specific PCR efficiency was used to calculate the expression of target genes relative to the expression of β-actin reference gene.

Statistics Analysis

Data are expressed as means±SEM. Statistical differences among the means of the various groups were analyzed by one-way analysis of variance of SPSS 13.0 software (SPSS Inc., Chicago, USA). Results were considered to be statistically significant at $P<0.05$.

Results

Effects of Chromium and/or Insulin Supplementation on Glucose Uptake and Metabolism

It has been shown that the reduced glucose concentration of the culture medium was higher ($P<0.05$) when the cells were cultured in the presence of insulin (Fig. 1). In the presence of insulin, ΔC of glucose of cells created with CrPic and CrSP were both higher ($P<0.05$) than treated with insulin only. The difference of ΔC of glucose between various chromium forms was not significant ($P>0.05$) when cells were treated in the absence of insulin.

Effects of Chromium and/or Insulin Supplementation on IR Gene Expression

Compared with the cells treated with neither Cr nor insulin, IR mRNA levels were upregulated ($P<0.05$) by 30%, 42%, 49%, and 88%, 101%, 142%, 142% when treated with CrCl, CrPic, and CrSP in the absence and/or presence of insulin, respectively (Fig. 2a). Compared with the cells treated with CrCl, in the presence or absence of insulin, IR gene expression levels were higher ($P<0.05$) when cultured with CrPic and CrSP. The difference between treatment of CrPic and CrSP was not significant ($P>0.05$).

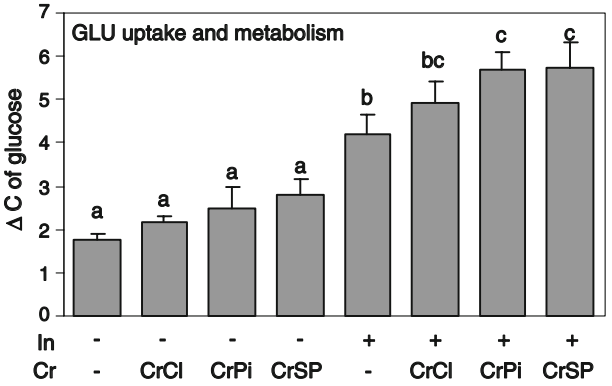

Fig. 1 Effects of chromium and/or insulin supplementation on glucose uptake and metabolism in cultured rat L6 skeletal muscle myoblasts. Skeletal muscle cells were left untreated or treated with 0.5 μM different forms of chromium in serum-free DMEM in the presence or absence of insulin (100 nM) for 72 h. The value of ΔC was the reduced glucose concentration (mM) of the culture medium relative to the original. ΔC could be regarded as the uptake and metabolized glucose by the muscle cells. Data are presented as the means± SEM, n=4. Different *superscripts* indicate significant difference (P<0.05)

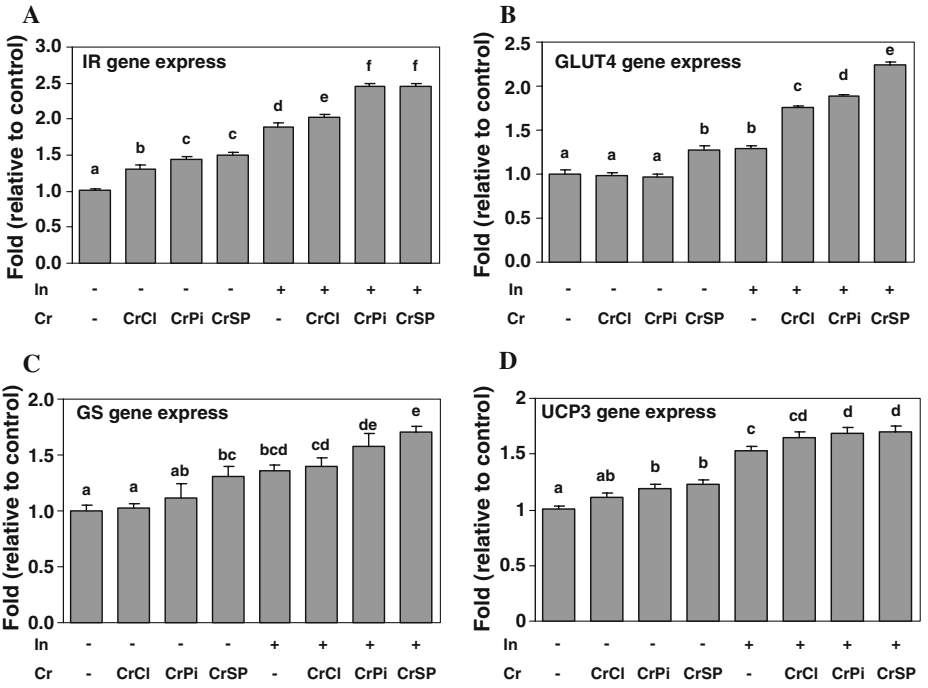

Fig. 2 Effects of various chromium forms and/or insulin on express of IR (**a**), GLUT4 (**b**), GS (**c**), and UCP3 (**d**) gene in L6 cells. Skeletal muscle cells were left untreated or treated with 0.5 μM different forms of chromium in serum-free DMEM in the presence or absence of insulin (100 nM) for 72 h. The mRNA levels of IR (**a**), GLUT4 (**b**), GS (**c**), and UCP3 (**d**) were analyzed by RT-PCR as described in the text. Data are presented as the means±SEM, n=3. Different *superscripts* indicate significant difference (P<0.05)

Effects of Chromium and/or Insulin Supplementation on GLUT4 Gene Expression

The results shown in Fig. 2b demonstrate that in the absence of insulin, the GLUT4 mRNA levels were not affected ($P>0.05$) by supplementation of CrCl and CrPic and upregulated ($P<0.05$) by CrSP and in the presence of insulin. There was no significant ($P>0.05$) difference between cells treated with CrSP or insulin only. Compared with the cells treated with insulin only, GLUT4 mRNA levels were upregulated ($P<0.05$) by 36%, 46%, and 74% when cultured with CrCl, CrPic, and CrSP in the presence of insulin, respectively. The difference between them was significant ($P<0.05$).

Effects of Chromium and/or Insulin Supplementation on GS Gene Expression

In the absence of insulin, GS mRNA levels were not affected ($P>0.05$) by supplementation of CrCl and CrPic and upregulated ($P<0.05$) by CrSP and in the presence of insulin (Fig. 2c). The difference between cells treated with CrSP or insulin only was not significant ($P>0.05$). Compared with the cells treated with CrSP only, GS mRNA levels were upregulated ($P<0.05$) by 20% and 30% when cultured with CrPic and CrSP in the presence of insulin, respectively. The difference between the two treatments was not significant ($P>0.05$). GS mRNA levels of the cells treated with both CrSP and insulin were higher ($P<0.05$) than the other treatments, except the cells treated with both CrPic and insulin.

Effects of Chromium and/or Insulin Supplementation on UCP3 Gene Expression

It can be seen from the Fig. 2d that the UCP3 mRNA levels were all upregulated ($P<0.05$) by more than 20% when cultured with different forms of Cr and/or insulin, except that of treated with CrCl only. Compared with the cells cultured without insulin or with insulin only, supplementation of CrPic and CrSP improved UCP3 gene expression significantly ($P<0.05$). There was no significant ($P>0.05$) difference among the three kinds of Cr when the cells were treated in the absence or presence of insulin.

Discussion

Chromium is an essential nutrient involved in the uptake and metabolism of glucose. Chromium has been shown to improve insulin-stimulated glucose uptake in cultured cells sensitive to insulin [7, 11, 12]. In the present study, chromium did not have a significant effect on basal glucose uptake and metabolism in the absence of insulin. These results demonstrate that chromium potentiates insulin-stimulated but not basal glucose uptake, consistent with a previous report [5].

It has been suggested that chromium enhances insulin binding, insulin receptor number, insulin internalization, and cell sensitivity [13]. The enhancement of insulin action by chromium is associated with phosphorylation of insulin receptor substrate-1 (IRS-1) [11] and phosphatidylinositol 3-kinase (PI 3-kinase) [14]. In the present study, cells treated singly with different forms of chromium could improve the IR gene expression, and these effects could be enhanced in the presence of insulin. The effects of CrPic and CrSP were better than that of the CrCl. Although the number of insulin receptors in these experiments was not directly measured, the results suggest that receptor number could be greater in the cells grown in the presence of chromium because the mRNA levels of IR were greatest in these cells.

Under insulin-stimulated conditions, skeletal muscle is quantitatively the most important tissue responsible for whole body glucose uptake in humans [15–17] and rodents [18]. GLUT4 is important in both the regulation of glucose uptake in skeletal muscle and the maintenance of whole body glucose homeostasis [19–27]. In muscle and adipose tissues, insulin stimulates the removal of circulatory glucose by regulating the subcellular trafficking of the glucose transporter GLUT4. Glucose transport is rate-limiting for glucose metabolism. In the basal state, GLUT4 cycles continuously between the plasma membrane and an intracellular compartment(s), with the majority of the transporter residing within the cell interior [28]. Activation of the IR by insulin triggers a large increase in the rate of GLUT4 vesicle exocytosis and a smaller but important decrease in the rate of internalization by endocytosis [29–31]. The overall insulin-dependent shift in the dynamics of GLUT4 vesicle trafficking results in a net increase of GLUT4 protein levels in the plasma membrane that amplify the cellular uptake of glucose. In the present study, the GLUT4 mRNA levels of muscle cells can be upregulated when treated singly with CrSP or insulin. Insulin can increase the mRNA levels of GLUT4, and this effect can be enhanced by the addition of different forms of chromium; that of CrSP is the highest, followed by CrPic, then that of CrCl. Further studies in this regard need to be done.

The magnitude of glycogen synthesis is regulated by glucose availability and glycogen synthase activity; both processes can be activated by insulin via independent upstream signaling pathways. Insulin is thought to enhance muscle GS activity through the activation of protein kinase B (PKB/Akt) and subsequent phosphorylation and deactivation of glycogen synthase kinase 3 (GSK3), which subsequently promotes dephosphorylation of GS at a cluster of COOH-terminal serine residues. Insulin promotes dephosphorylation and activation of GS by inactivating glycogen synthase kinase (GSK) 3 through phosphorylation [32]. The effect of Cr on GS genes express in muscle cells was first determined in this study. Insulin can improve the gene expression of GS, and this effect can be enhanced by the addition of chromium, especially with the form of CrSP. Chromium increases phosphoinositide 3-kinase activity, glucose uptake, and glycogen content in cultured human skeletal muscle cells [33]. Besides its involvement in the activation of GLUT4 vesicles, Akt can phosphorylate glycogen synthase kinase 3, which is an essential step in the activation of GS, the enzyme involved in glycogen synthesis. The present study indicates that Cr may improve insulin sensitivity also by upregulating the mRNA levels of GS. Further studies will determine whether Cr improves GS activity and GS protein content.

UCP3 plays a role in regulating glucose metabolism. UCP3 stimulates glucose transport and GLUT4 translocation to the cell surface in skeletal muscle cells by activating a phosphoinositide-3-kinase-dependent pathway [9]. A positive correlation between UCP3 mRNA and glucose utilization in lean NIDDM patients has been reported. A reduced UCP3 mRNA expression has been reported in the muscle of type 2 diabetic patients [34]. Although UCP3 knockout mice appear to have normal glucose homeostasis [35, 36], high level overexpression of either UCP1 or UCP3 in the muscle of transgenic mice results in increased energy expenditure, resistance to diet-induced obesity, and increased glucose tolerance and insulin sensitivity [37, 38]. In this study, exposure of cells to chromium increased the basal and insulin-stimulated mRNA levels of UCP3.

The absorption and utilization of Cr is dependent on its status in the gastrointestinal tract. Under physiological pH, in the inorganic form, such as chromium chloride and chromium nitrate, trivalent chromium has a strong tendency to undergo olation and form insoluble macromolecular chromium oxide [39]. As to CrSP, the small peptides serve as powerful ligands to protect against the olation process or to deolate precipitates by replacing the hydroxy groups and thus solubilizing the complexes. The organic forms of Cr,

CrPic, and CrNic, the other two kinds of chelates (chromium chelated with picolinic acid and nicotinic acid, respectively), are very stable [40]. The CrPic is the most popularly used dietary supplement in animals and humans. However, recent reports have indicated that the picolinate ligand may 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 [2, 3]. Recently, it was reported that chromium chelated with amino acid appears to be the most effective [4, 5] and did not cleave DNA under physiological conditions [5]. In most of the results of our present study, the difference with chromium chloride relative to CrPic and CrSP was quite small, while as to the effects of chromium supplementation on GLUT4 gene expression, CrSP had greater ($P<0.05$) effect than that of CrPic in the presence of insulin.

This study confirmed that CrSP had better effects than that of CrPic and CrCl, which is consistent with the recent previous similar report [5]. Emerging evidence has shown that the biologically active form of chromium is a chromium–oligopeptide complex [1], which further justifies the use of organic (especially that of oligopeptide, or small peptide) chromium complexes as biomimetic chromium supplements.

Conclusion

In conclusion, the major findings of this study are: (1) Chromium, especially the form of CrSP, enhances insulin-stimulated glucose uptake and metabolism in skeletal muscle cells. (2) chromium, especially at the form of CrSP, upregulates mRNA levels of IR, GLUT4, GS, and UCP3, (3) and these effects are independent of and enhanced in the presence of insulin. (4) The upregulated mRNA levels of UCP3 may play an important role in the physiological function of chromium, which needs further study.

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