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Toxicology

Mechanism of insulin-like effect of chromium(III) ions on glucose uptake in C2C12 mouse myotubes involves ROS formation

Maciej Lipko*, Bogdan Debski

Department of Physiological Science, Faculty of Veterinary Medicine, Warsaw Agricultural University, Poland

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ABSTRACT

Chromium is considered a trace element which improves glucose tolerance, but mechanism accounting for this insulin-like action is not recognized. The main purpose of this study was to examine the role of reactive oxygen species (ROS) in chromium and insulin stimulated glucose transport using antioxidants. Effect of chromium ions on phosphatases, enzymes involved in inhibition of insulin signaling was also investigated. Experiments were performed *in vitro* on C2C12 mouse myotubes. ROS level was measured with the use of confocal microscope and 2',7' dichlorodihydrofluorescein diacetate (DCFH-DA). Glucose metabolism was assayed by the measurement of 2-[^{3}H]-deoxyglucose uptake. Cr^{3+} ions and insulin treatment caused significant increase of ROS formation and also stimulated glucose uptake in C2C12 cells in concentration dependent manner. Antioxidants (L-ascorbic acid and N-acetyl cysteine 100 μM) and DPI (diphenyleneiodonium-NADPH oxidase inhibitor, 10 μM) abolished insulin- and Cr-inducted glucose transport. Our results confirm the hypothesis that the ROS are integral part of insulin signaling pathway and that the insulin mimetic effect of Cr^{3+} ions depends on the antioxidant status of the cells. Surprisingly, chromium treatment resulted in increased activity of membrane phosphatases.

1. Introduction

Trivalent chromium is known to enhance insulin action [1–3] and is necessary for optimal carbohydrate and lipid metabolism. It was observed that chromium supplementation reversed glucose intolerance in patients receiving parenteral nutrition [4]. Supplementation of rats with water containing Cr^{3+} ions (2 mg/L) resulted in enhanced glucose and glycogen metabolism and increased incorporation of amino acids into tissue protein [5]. Involvement of chromium in glucose metabolism is supported by observation that in patients suffering type 2 diabetes, enhanced Cr excretion occurs [6,7] and dietary chromium deficiency has been shown to increase the risk of insulin resistance [8].

Clinical trials have demonstrated that Cr supplementation helps people with NIDDM (non-insulin dependent diabetes mellitus) [9,10]. It improves glucose metabolism in rats with streptozotocin-inducted diabetes [11,12] and in obese, insulin-resistant rodents [13,14]. Since chromium deficiency is connected with insulin resistance and cardiovascular diseases, its dietary adequacy have significant implications [15]. Support for this hypothesis comes from observations that in obese rats chromium picolinate treatment lowers plasma cholesterol and enhances insulin level and tissues sensitivity [16]. Diabetes is a leading cause of morbidity and mortality in the developing countries [17]. Current insulin mimicking drugs: thiazolidinediones and biguanides have disadvantageous side effects and it is why there is a growing need for pharmacological agents that improve the sensitivity of insulin. In the previous years the studies aimed mainly on effects of chromium deficiency or supplementation. The mechanism(s) responsible for its action remains unsolved. In 1957 the substance isolated from porcine kidney was found to reverse glucose intolerance and was named as glucose tolerance factor (GTF) [18], but finally it has been shown to be an artifact, generated during its isolation. At present, the candidate for the biologically active form of chromium is an oligopeptide, low-molecular-weight chromium binding substance (LMWCr), found mainly in the liver of mammals, also known as chromodulin [19]. Davis and Vincent demonstrated in isolated rat adipocytes that LMWCr stimulates 8-fold activity of receptor tyrosine kinase (RTK) [20]. One way of regulation of insulin signal transduction is reversible oxidation of protein tyrosine phosphatases (PTPs) by ROS [21]. Chromium exists on every oxidation state from 0 to +6 and is involved in red/ox reactions in biological systems. The present study investigated the effect of Cr^{3+}

Abbreviations: DCFH DA, dichorodihydrofluorescein diacetate; DCFH 2′,7′, dichorodihydrofluorescein; DCF 2′,7′, dichorofluorescein; DMEM, dulbecco modified eagle's medium; DOG, deoxyglucose; DPI, diphenyleneiodonium; GTF, glucose tolerance factor; GLUT4, glucose transporter isoform 4; HS, horse serum; FBS, fetal bovine serum; IR, insulin receptor; IRS, insulin receptor substrate; NIDDM, non-insulin dependent diabetes mellitus; PBS, phosphate buffered saline; PTPs, protein tyrosine phosphatases; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; SHPTP2, src homology 2 protein tyrosine phosphatase

* Corresponding author.
E-mail address: lipko.maciej@gmail.com (M. Lipko).

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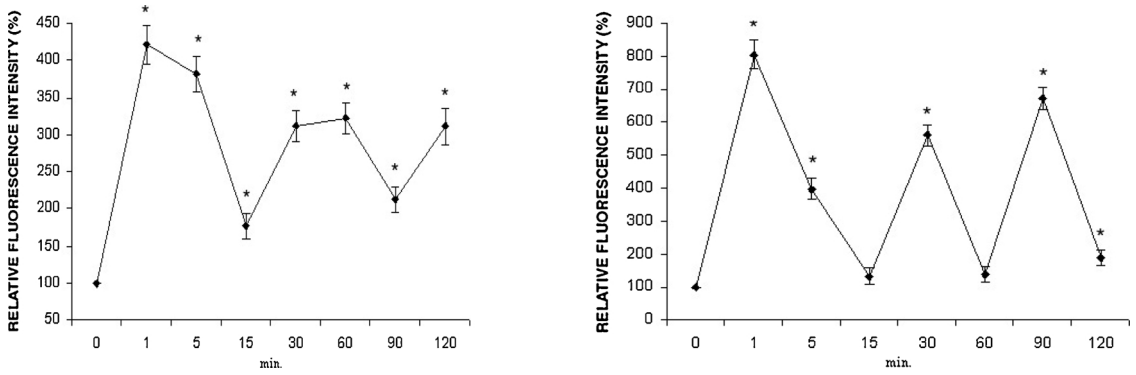

Fig. 1. Time-dependent effect of insulin (100 nM) – [A] or Cr^{3+} ions (10 μg/L) – [B] on intracellular synthesis of reactive oxygen species (ROS).

and insulin on ROS formation and also phosphatases activity in myotubes formed by mouse C2C12 cells. It was previously shown that insulin signaling pathway requires multiple phosphorylation/dephosphorylation events [21] which are modulated by ROS [22,23]. It is known that low concentration of H_2O_2 stimulates glucose transport and GLUT4 translocation in skeletal muscle cells [24]. Additionally, we investigated the influence of chromium and insulin on glucose uptake in the presence and absence of antioxidants in order to examine the role of ROS in the insulin signaling pathway.

2. Materials and methods

2.1. Cell culture

Experiments were performed *in vitro* with the use of mouse C2C12 myogenic cells. Cells were grown in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 20% fetal bovine serum (FBS) and antibiotics (penicillin-streptomycin (50 IU/ml) + fungizone (2.5 μg/ml)) in atmosphere of 5% CO_2/95% humidified air at 37 °C. Confluent culture was differentiated in DMEM enriched with 2% horse serum (HS) for four days.

2.2. Assay of glucose transport

C2C12 myoblasts were grown on 24-well plates. After 4 days of culture in the differentiation medium the formation of myotubes was well defined. The myotubes were incubated in serum free DMEM containing Cr^{3+} ions administered in a form of $CrCl_3$ (0,01 ÷ 100 μg/L), or insulin (20 ÷ 100 nM), in the presence/absence of antioxidants: L-ascorbic acid, N-acetyl cystine (100 μM), and diphenyleneiodonium DPI (NADPH oxidase inhibitor – 10 μM). Monolayers were rinsed with HEPES buffer (20 mM HEPES, 140 mM NaCl, 5 mM KCl, 2,5 mM $MgSO_4$, 1 mM $CaCl_2$, pH 7,4), and then assayed for [^{3}H] 2-deoxy glucose uptake. Uptake of glucose was initiated by the addition of 1 ml of HEPES buffer with 1 mCi 2-[^{3}H]-deoxy glucose (ICN) and 1 mM 2-deoxy glucose for 10 min for each well. Radioactivity was determined after cell lysis in 0,05 M NaOH, followed by scintillation counting, using HiSafe (Pharmacia) liquid scintillation cocktail in Packard TriCarb counter.

2.3. Assay of intracellular ROS

Cells were incubated in darkness for 10 min in PBS containing 5 μM DCFH-DA (2′-7′ dichorodihydrofluorescein diacetate), after above mentioned stimulation with chromium or insulin. The substrate was converted by intracellular esterases to DCFH (2'-7' dichorodihydrofluorescein), which was then oxidized by H_2O_2 to highly fluorescent DCF (2'-7' dichorofluorescein). Fluorescence was measured with an excitation wavelength of 488 nm and emission 515 ÷ 540 nm on confocal microscope FluoView 500. Image was collected by single rapid scan with identical parameters.

2.4. Assay of phosphatases activity

Activity of phosphatases was assayed by hydrolysis of synthetic substrate p-nitrophenol phosphate (p-NPP). Samples were preincubated for 5 min in 37 °C in reaction buffer (250 mM HEPES, 50 mM ditiothreitol, 25 mM EDTA). The reaction was started by adding 0,02 ml 20 mM p-NPP, carried out for 30 min at 37 °C, and stopped by adding 0,3 ml of 0,5 M NaOH. The absorbance of the sample was measured at 410 nm.

2.5. Cell fractionation

Myotubes treated experimentally were washed with PBS and mechanically scraped in a buffer containing 0,32 M sucrose, 10 mM Tris (pH 7,5), 5 mM EGTA and 1 mM EDTA. Nuclei were removed by centrifugation at 2000 × *g* at 4 °C for 10 min. The membrane fraction was sedimented by an additional centrifugation at 15,000 × *g* at 4 °C for 60 min, resuspended in buffer containing 250 mM HEPES (pH 7,2), 140 mM NaCl, PMSF and leupeptine. Twenty micrograms of membranes from control or treated cells were used in the assay.

2.6. Statistics

All experiments were performed in triplicate wells for each condition and repeated three times. Data represent mean ± SEM. Statistically significant differences were determined with Student's *t*-test and are indicated with: asterisk (*) $P < 0.05$ for differences between control vs. chromium or insulin, hashtag ($^{\#}$) $P < 0.05$ for differences between chromium or insulin vs. chromium or insulin + antioxidant.

3. Results

Fig. 1 Insulin stimulation (100 nM) caused an increase of ROS formation and this effect reached maximum after 1, 30, 90 min of treatment, reaching 800%, 540% and 670% of control value, respectively (Fig. 1 a). Treatment of C2C12 cells with chromium ions also resulted in increased ROS formation and the maximal (400%) increase was seen after 1 min of stimulation. Substantial increase of ROS formation was observed also after 30–60, and 120 min of treatment, reaching 300% and 340% of control value, respectively (Fig. 1 b).

Fig. 2 Results of time dependent effect of chromium on glucose transport are presented on Fig. 2. Chromium ions at concentration 10 μg/L ($CrCl_3$) enhanced glucose transport in time dependent manner. After 6 h of incubation with Cr^{3+}, uptake of 2-deoxyglucose (DOG) reached maximum (136% of control), and started to decline.

Fig. 3 Effects of various concentrations of chromium ions (0,01–100 μg/L) on ROS formation are shown on Fig. 3. Cr^{3+} ions