SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 3: 347-353, 2010

Evaluation of insulin binding and signaling activity of newly synthesized chromium(III) complexes *in vitro*

PAWEL MACKOWIAK[1], ZBIGNIEW KREJPCIO[2], MACIEJ SASSEK[1], PRZEMYSLAW KACZMAREK[1], IWONA HERTIG[1], JOANNA CHMIELEWSKA[1], TATIANA WOJCIECHOWICZ[1], DAWID SZCZEPANKIEWICZ[1], DARIA WIECZOREK[3], HENRYK SZYMUSIAK[3] and KRZYSZTOF W. NOWAK[1]

Departments of [1]Animal Physiology and Biochemistry, and [2]Human Nutrition and Hygiene, Poznan University of Life Sciences; [3]Department of Technology and Environmental Protection, Poznan University of Economics, Poznan, Poland

Received November 12, 2009; Accepted January 19, 2010

DOI: 10.3892/mmr_00000264

Abstract. In the present study, the influence of chromium(III) complexes (acetate, chloride, glycinate, histidinate, lactate and propionate) on insulin binding and signal transduction [phosphorylation of tyrosine and serine in the insulin receptor substrate (IRS)-1] was investigated *in vitro* using three experimental models: isolated rat liver membranes and cultured mouse C2C12 myoblasts or 3T3-L1 preadipocytes. The examined complexes did not elevate the binding of insulin to the liver membranes. Moreover, chromium histidinate, lactate, acetate and propionate complexes diminished the specific binding of insulin. Simultaneously, chromium chloride, which did not significantly elevate insulin binding, increased the number of membrane accessible particles of the insulin receptors. However, it was accompanied by slightly diminished affinity of the receptor to the hormone. Chromium acetate and propionate significantly diminished the binding capacity of the low-affinity insulin receptor class. Investigations with the myoblast cell line C2C12 and preadipocyte cell line 3T3-L1 did not allow differentiation of the influence of the examined complexes on insulin binding. Immunodetection of phosphorylated forms of IRS-1 showed that the chromium compounds modulated the transduction of the insulin signal. Chromium glycinate, acetate and propionate decreased the amount of IRS-1 phosphorylated at serine. Since it is generally thought that phosphorylation of serine in IRS-1 may moderate insulin action, the above mentioned chromium complexes may, in this way, enhance insulin effects inside target cells. Phosphorylation of tyrosine in IRS-1, which acts as a stimulatory signal for further steps of insulin action, was elevated after the incubation of 3T3-L1 cells with insulin. Chromium supplementation did not additionally intensify this process. However, in the absence of insulin, chromium glycinate and acetate slightly elevated the level of IRS-1 phosphorylated at tyrosine. This fact may be important *in vivo* at low levels of insulin in blood. The results indicate that the action of chromium(III) complexes involves a direct effect on the number of receptors accessible to insulin, their affinity to the hormone and the modulation of the signal multiplying proteins by their phosphorylation.

Correspondence to: Professor Krzysztof W. Nowak, Department of Animal Physiology and Biochemistry, Poznan University of Life Sciences, 35 Wolynska Street, 60-637 Poznan, Poland
E-mail: kwnowak@au.poznan.pl

Key words: chromium(III) complexes, insulin signaling, insulin receptor, insulin receptor substrate-1

Introduction

Insulin is one of the main factors regulating the homeostasis of organisms. It is well known that disturbances of insulin action play a crucial role in many diseases and pathogenic conditions. It is necessary to mention pandemic disorders such as diabetes mellitus type 2, obesity, arteriosclerosis, hypertension and metabolic syndrome – the set of symptoms related to disturbances of homeostasis with insulin resistance (1,2). Insulin resistance is the pre-diabetic state, closely associated with obesity, and is defined as the weaker or stronger inability of tissues to respond to insulin (3). Inherent connections between resistance to insulin and obesity have been confirmed, suggesting that a proper pattern of nutrition and a healthy lifestyle are crucial to prevention (4). However, in the case of disease, medical therapy is necessary, particularly the use of pharmacological drugs that enhance tissue sensitivity to insulin. Chromium (Cr) compounds, often used as a dietary supplement, are candidates for improving metabolism and diminishing disturbances observed in diabetes (5-7). Researchers investigating diabetes focus on several areas: the regulation of insulin secretion, its circulation, its binding to the receptor, as well as transduction of the signal inside target cells. Insulin is a hormone with pleiotropic functions that influence mitogenesis and gene expression and affect the metabolism of carbohydrates, lipids and proteins (8). The first and necessary step of insulin action on cells is its binding to membrane receptors commonly present in organisms (9). Diverse tissues possess different numbers of receptors accessible to insulin that are classified in two functional pools: high- and low-affinity insulin receptors (HAIRs and LAIRs). Simultaneously, the number and affinity of the insulin receptors differ between tissues (10) and are related to various

aspects (diet, age, temperature and sickness) of the physiological condition of the organism (11-14). After binding to the extracellular subunit, the signal is transmitted inside target cells. The first step is autophosphorylation of the β-subunit and interaction with proteins from the insulin receptor substrate (IRS) family (15,16).

IRS-1 to -4 are known to be responsible for further transduction pathways (17). The principal role in cellular signaling is played by IRS-1, which is prevalent in muscles, and by IRS-2, which is expressed in liver, muscles and adipose tissue. Moreover, it has been found that IRS-3 and -4 are expressed in white adipose tissue, thymus, brain and kidneys. However, these proteins play a rather marginal role in insulin signaling (18,19). There are two dominant modes of IRS action, depending on the place where IRS is phosphorylated. The phosphorylation of tyrosine generally has an activating influence on the signal transduction cascade. By contrast, the phosphorylation of serine may suppress it. The occurrence of both types of IRS phosphorylation enables complex regulation of the insulin signal transduction system (20-25).

Cr is thought to be an essential trace element for mammals, allowing the maintenance of proper carbohydrate and lipid metabolism (24). Diabetic patients may show low levels of Cr in their serum and Cr deficiency. Supplementation of the diet with Cr may be beneficial for improving insulin action (7). However, the molecular mechanisms of Cr action are poorly understood.

Several studies have demonstrated the beneficial effects of supplemental trivalent Cr in subjects with reduced insulin sensitivity and in those with type 2 diabetes mellitus, with no documented signs of toxicity (7). However, recent studies have questioned the safety of certain supplemental trivalent Cr complexes (26). Comparative studies of chromium(III) picolinate and niacin-bound chromium(III), two popular dietary supplements, have revealed that chromium(III) picolinate produces significantly more oxidative stress and DNA damage (27-30). Recently, chromium picolinate has been shown to be mutagenic in some *in vitro* tests, and picolinic acid moiety appears to be responsible for that effect (27). The safety of chromium(III) is largely dependent on the type of ligand, thus adequate clinical studies are warranted to demonstrate the safety and efficacy of chromium(III) for human consumption.

The $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ cation has been studied and proposed as an alternative supplemental source of Cr. This form of chromium(III) is absorbed with greater than 60% efficiency at nutritional supplement levels and with greater than 40% efficiency at pharmacological levels, which is an order of magnitude greater than the absorption of $CrCl_3$, chromium nicotinate or chromium picolinate, the currently marketed nutritional supplements (31). The difference in the degree of absorption is readily explained by stability and solubility which, together with collected data on toxicity, can be regarded as the most relevant criteria for quality.

These findings indicate an urgent need to search for safer chromium(III) derivatives that can be used as a source of Cr for dietetic and therapeutic purposes. Therefore, the aim of this study was to synthesize four chromium(III) complexes with carboxylic acids (acetic, glycine, lactic and propionic), and to compare the action of these new compounds with those currently used in dietary supplements, such as chromium(III) chloride and chromium(III) histidinate, on insulin binding to rat liver membranes and the membranes of *in vitro* cultured mouse C2C12 myoblasts and 3T3-L1 preadipocytes. Moreover, tyrosine and serine phosphorylation in IRS-1 was investigated to acquire further information concerning the modulation of insulin signal transduction by chromium(III).

Materials and methods

To investigate the influence of different chromium(III) compounds on insulin signal reception at the stage of insulin binding to its receptor and on the level of intracellular post-receptor pathways, we used isolated rat liver membranes, the mouse myoblast cell line C2C12 and mouse 3T3-L1 preadipocytes.

Usage of chromium(III) complexes. Four different trinuclear chromium(III) complexes with carboxylic acids (acetate, glycinate, lactate and propionate) in the form of nitrate salts (general chemical formula $[Cr_3O(acid)_6(H_2O)_3]^+NO_3^-$) were synthesized in our laboratory according to the method described previously by Earnshaw and Lewis (32). The products were recrystallized from water and then desiccated under vacuum at 100˚C. Their physicochemical characteristics were obtained by selected instrumental methods, UV-VIS and IR spectroscopy, and thermal analysis. Thermal stability of the samples was determined using DTA and TGA methods (module DTA-50; Shimadzu). UV-VIS absorption spectra were recorded using a Genesys 2 spectrophotometer. IR spectra were recorded (in KBr) using a Specord M-80 spectrophotometer. Details of these methods have been described in our previous studies (33-35). For comparative studies, we used two other Cr salts: chromium chloride ($CrCl_3 \cdot 6\ H_2O$) purchased from Sigma (St. Louis. MO, USA), and chromium histidinate provided courtesy of Dr R.A. Anderson (Nutrition Research Center, US Department of Agriculture, ARS, Beltsville, MD, USA). Both compounds are currently used in dietary supplements containing Cr that are marketed in many countries. The contents of elemental Cr, chromium acetate, glycinate, lactate, propionate complexes and chloride as well as histidinate in the compounds were determined by the AAS method and were 25.4, 17.3, 17.8, 20.4, 19.5 and 7.80%, respectively. Aqueous stock solutions of these compounds containing 10 mM Cr^{3+} were prepared for all experiments. The concentrations were assured by quantitative elemental analysis using the AAS method (spectrometer AAS-3 with BC correction; Zeiss).

Biological material. Mouse C2C12 and 3T3-L1 cells were cultured under standard conditions (37.0˚C; 95% air/5% CO_2) in DMEM medium with L-glutamine and glucose (Gibco, Grand Island, NY, USA) supplemented with 20% FBS (Sigma) and antibiotics (Gibco). Donors of liver cell membranes were male Wistar rats (body weight 180-220 g) housed in a temperature-controlled room at 20.0˚C and maintained on a standard laboratory rat chow with free access to food and water.

Insulin binding. For 24 h before investigation, C2C12 and 3T3-L1 cells were incubated in the presence of 1, 10 and 100 μM chromium(III) complexes. Rat liver membranes

Table I. *In vitro* effect of Cr(III) complexes on specific ^{125}I-insulin binding (B_{max}%) to isolated rat membranes.

Cr(III) complex	Cr(III) concentration (μmol/l)	B_{max}%
None (control)	0	30.3±0.8
Chloride	1	30.9±0.6
	10	29.1±0.6
	100	32.0±1.4
Glycinate	1	28.4±1.9
	10	29.8±1.8
	100	33.3±1.8
Histidinate	1	28.8±2.0
	10	29.2±1.8
	100	21.5±1.9[a]
Lactate	1	26.5±1.9
	10	27.6±2.0
	100	23.9±1.6[a]
Acetate	1	27.6±1.6
	10	26.4±1.0
	100	24.5±1.4[a]
Propionate	1	24.1±1.8[a]
	10	24.6±1.4[a]
	100	23.4±0.8[a]

Results calculated per 1 mg of membrane protein (mean ± SEM of eight experiments). [a]Statistically significant differences in comparison to the control ($p \leq 0.01$).

and the cultured mouse cells were isolated according to the method of Havrankowa *et al* (36) and dissolved in 50 mM Tris-HCl buffer (pH 7.4) containing 0.1% bovine serum albumin (BSA). Next, they were incubated for 18 h at 4.0˚C with 0.05 nM porcine receptor grade ^{125}I-insulin (Perkin Elmer), unlabelled human insulin (Novo Nordisk A/S) and with different concentrations of Cr compounds. Non-specific binding was determined in the presence of 10 μM unlabelled insulin. Bound and free fractions of insulin after incubation were separated by centrifugation at 30,000 x g for 8 min. The radioactivity of the pellets was measured using a gamma-counter (Wallac). The binding capacity of the receptors and dissociation constants (Kd) was calculated by Scatchard's method using the Ligand-PC v.2.7 computer program (37).

Investigation of IRS-1 phosphorylation in 3T3-L1 cells. Cells were cultured in the presence of a 10 μM concentration of the four tested compounds (the medium dose used in previous ligand binding experiments was chosen for the insulin signaling assay) and then exposed to 100 nM insulin for 60 min. After activation of the insulin receptor, incubation medium was drained out and the cells were washed twice with PBS, suspended in NP-40 lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40 and 2 mM EDTA), constantly agitated for 30 min on ice and centrifuged at 4˚C. All solutions were ice cold, and a freshly prepared protease-phosphatase inhibitor cocktail (Roche Applied Science, USA) was added to the lysis buffer. The supernatant with extracted proteins was gently aspirated and transferred to pre-cooled microfuge tubes. Following the determination of isolation efficiency (Bradford assay), equal amounts (100 μg) of extracted proteins were subjected to immunoprecipitation with the anti-IRS-1 antibody (Abcam, USA) using the Protein G Immunoprecipitation kit (Sigma-Aldrich) according to the manufacturer's protocol. After immunoprecipitation, samples were divided, and an equal amount of each sample was resolved on two separate 12% PAA gels. Next, immunoprecipitated proteins were blotted on a PVDF membrane according to the standard protocol. In order to verify and compare the level of tyrosine and serine IRS-1 phosphorylation, immunodetection with phospho Y869 IRS-1 (ab47776; Abcam, USA) and phospho S1101 IRS-1 (ab55343; Abcam, USA) antibodies was performed using Western blotting. The Local Ethics Committee for Experiments on Animals approved the experimental protocol.

Statistical analysis. Results were averaged per experimental group. Statistical comparison of the data was carried out using ANOVA, followed by the Duncan multiple range test.

Results

At the start of the performed investigations, we focused on the influence of various concentrations of different chromium(III) complexes on the specific binding of insulin to its liver membrane receptor. We found that the compounds did not significantly elevate specific insulin binding (Table I). In the case of chromium chloride and glycinate, ^{125}I-insulin binding (B_{max}%) was maintained at the level characteristic for non-treated membranes; however, for the applied maximal concentration (100 μM), B_{max}% was slightly elevated. Chromium histidinate, lactate, acetate and propionate complexes diminished insulin binding. This was particularly evident for maximal doses of chromium histidinate, lactate, acetate, and for all concentrations of propionate complex. These changes were statistically significant. Four compounds, chromium chloride and glycinate, acetate and propionate complexes, were selected for further study. Liver membranes were treated with a 10 μM concentration of the Cr compounds, and full analysis of insulin binding was carried out. These compounds altered the curvilinear shape of the obtained displacement curves and Scatchard's plots in comparison to the control (plots not shown) indicating alterations in insulin binding in the presence of the Cr complexes. The final results of the Scatchard's analysis are presented in Table II. The incubation of membranes with chromium chloride significantly increased the binding capacity of accessible HAIRs and LAIRs. Glycinate did not change these parameters in comparison to the control, whereas acetate and propionate did not influence HAIRs but diminished the binding capacity of LAIRs. None of the investigated complexes significantly changed the affinity of HAIRs and LAIRs to insulin (calculated as dissociation constant; Kd).

In the next step, we repeated the investigations using the myoblast cell line C2C12 and the preadipocyte cell line 3T3-L1. The results (Table III) indicated that the obtained

Table II. *In vitro* effect of a 10-μM concentration of Cr(III) complexes on rat liver insulin receptor and its affinity to insulin.

	Control	Chloride	Glycinate	Acetate	Propionate
HAIR					
B_{max} (fmol/mg)	72±9	124±11[a]	87±5	67±9	60±9
Kd (pmol/l)	163±24	240±47	185±28	147±43	142±23
LAIR					
B_{max} (pmol/mg)	38±1	105±5[a]	41±7	11±2[a]	17±2[a]
Kd (nmol/l)	166±30	226±15	204±15	110±14	124±16

HAIR, high affinity pool of insulin receptor; LAIR, low affinity pool of insulin receptor; B_{max}, binding capacity of each pool of insulin receptor calculated per 1 mg of membrane protein; Kd, dissociation constant. Values represent the mean ± SEM of six experiments. [a]Statistically significant differences in comparison with control ($p<0.01$).

Table III. *In vitro* effect of different concentrations of the Cr(III) complexes on specific ^{125}I-insulin binding (B_{max}%) to C2C12 and 3T3-L1 cell membranes.

Cr(III) complex	Cr(III) concentration (μM)	C2C12 cells B_{max}%	3T3-L1 cells B_{max}%
None (control)	0	0.4±0.2	0.4±0.3
Chloride	1	0.4±0.3	0.4±0.3
	10	0.4±0.3	0.5±0.3
	100	0.4±0.3	0.4±0.2
Glycinate	1	0.5±0.2	0.4±0.3
	10	0.4±0.3	0.4±0.2
	100	0.4±0.2	0.5±0.3
Histidinate	1	0.4±0.3	0.3±0.2
	10	0.4±0.3	0.5±0.3
	100	0.4±0.3	0.4±0.4
Lactate	1	0.4±0.3	0.4±0.3
	10	0.4±0.3	0.4±0.3
	100	0.4±0.3	0.5±0.3
Acetate	1	0.4±0.4	0.4±0.3
	10	0.5±0.3	0.4±0.3
	100	0.5±0.3	0.4±0.2
Propionate	1	0.4±0.3	0.4±0.3
	10	0.5±0.3	0.4±0.3
	100	0.4±0.3	0.4±0.3

Results calculated per 10^6 cells represent the mean ± SEM of nine experiments. No changes were statistically significant.

specific binding was very similar in all cases, and the obtained values of B_{max}% were far too low to undertake a full Scatchard's analysis. This prevented the verification of the results obtained using the liver membranes. However, the cultured 3T3-L1 cells were useful for the investigation of the intracellular steps of insulin action. The results of phospho Y869 IRS-1 immunodetection are shown in Figs. 1 and 2 (representative of three performed assays). The phosphorylation of tyrosine in IRS-1 is responsible for the activation of insulin signal transduction. Insulin receptor activation and insulin signal transduction pathway functionality were clearly confirmed by the comparison of phospho Y869 IRS-1-specific signal intensity derived from the control and the insulin-treated cells (Fig. 1). Without the presence of insulin, none or very weak signals were detected. A strong signal was detected after incubation with the hormone. Simultaneously, no changes in IRS-1 tyrosine phosphorylation were induced by any of the tested Cr compounds in the presence of insulin (Fig. 1), whereas in the absence of the hormone, a slightly visible induction of phosphorylation was noted for chromium glycinate and

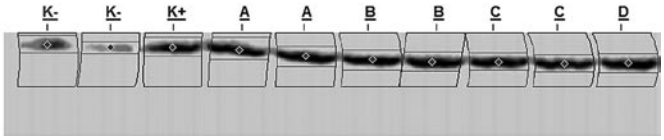

Figure 1. The effect of various chromium(III) complexes on the phospho Y869 IRS-1 relative level in 3T3-L1 cells. K-, cells incubated without insulin and chromium complexes; K+, cells incubated with 100 nM insulin in the absence of chromium complexes; A, B, C and D, cells incubated with 100 nM insulin and 10 μM chromium complexes (A, chloride, B, glycinate, C, acetate and D, propionate). Average ratio of band intensity after chromium to intensity of K+: A, 1.02; B, 0.98; C, 0.97; D, 1.15.

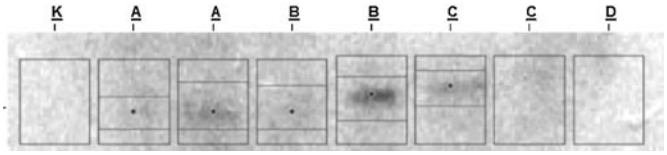

Figure 2. The effect of various chromium(III) complexes on the phospho Y869 IRS-1 relative level in 3T3-L1 cells without activation of the insulin receptor. Cells were incubated in the absence of insulin. K, without chromium complexes; A, B, C and D, cells incubated with 10 μM chromium complexes (A, chloride; B, glycinate; C, acetate; D, propionate).

acetate (Fig. 2). Additionally, the influence of serine phosphorylation in IRS-1 was found to be important for the action of the chromium(III) complexes on the pathways of target cells representing adipose tissue. Phosphorylation of serine may downregulate intracellular transmission of the insulin signal. Using phospho S1101 IRS-1 antibody, slightly diminished relative levels of phosphorylated IRS-1 were observed after the treatment of 3T3-L1 cells with chromium(III) acetate, and a considerably lower value after treatment with chromium(III) glycinate (Fig. 3). Relative values are provided in the figure legends.

Discussion

The beneficial action of certain chromium(III) compounds, particularly chromium picolinate and chloride, on metabolism, and their helpful role in the treatment of diabetes is well documented. However, new medications with anti-diabetic properties are still required. Additionally, the biochemical background of currently used Cr complexes does not seem simple and has not been fully elucidated. In this study, we tried to describe the mechanisms of action for some new as well as popularly used Cr compounds. Based on our studies of insulin receptor (9-14), we selected liver membranes as a suitable *in vitro* model for investigation. This model allowed us to estimate the specific binding of insulin, to calculate the binding capacity of receptors and to describe the kinetic parameters of binding in the absence or presence of the Cr complexes.

The binding of a hormone to a specific cell surface receptor is the first and obligatory step of action in the target tissue. This subsequently evokes a cascade of intracellular reactions. Thus, investigations of membrane receptors are important for the characterization of the sensibility of various cells to insulin. Similar to other studies (9-13), the typical curvilinear

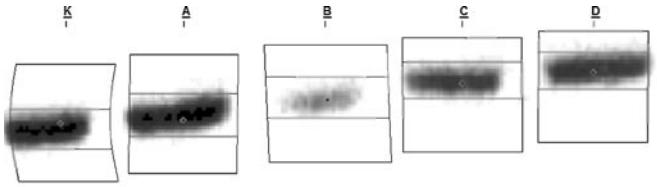

Figure 3. The effect of various chromium(III) complexes on the phospho S1101 IRS-1 relative level in 3T3-L1 cells. K+, cells incubated with 100 nM insulin in the absence of chromium complexes; A, B, C and D, cells incubated with 100 nM insulin and 10 μM chromium complexes (A, chloride; B, glycinate; C, acetate; D, propionate). Average ratio of band intensity after chromium to intensity of K+: A, 0.87; B, 0.58; C, 0.73; D, 0.93.

Scatchard plots and sigmoidal displacement curves observed during the present investigations and mathematical analysis (not presented) suggest, as is generally observed and accepted (38), that two physiologically different pools of insulin receptors exist: high-affinity (HAIRs) and low-affinity (LAIRs) high capacity receptors. Since liver, muscles and adipose tissue are, as previously shown (10-14,38), very important targets for insulin, we decided to use C2C12 myocytes and 3T3-L1 preadipocytes. These experimental models have previously been applied (39). The results showed that the chromium(III) complexes did not increase specific insulin binding (Tables I and III). On the contrary, histidinate, lactate, acetate, propionate, but not glycinate and chloride, significantly diminished the ability of the liver membranes to bind the hormone (Table I). This effect was dose-dependent and highly manifested at the highest dose of the employed compounds. Thus, the improvement in intracellular metabolism observed (also in diabetes) after treatment with different forms of chromium(III) (5-7,39-41) widely used for achieving optimal insulin function is most probably not a result of the elevated ability of the membranes of target organs and tissues to bind insulin. In the case of acetate and propionate, the reduced specific binding was probably due to the effect of the significantly diminished level of the LAIR pool (Table II). For chromium glycinate, the absence of changes in specific binding (Table I) was accompanied by non-significant statistical differences in the amount of HAIRs and LAIRs; however, the quantity of both classes of receptors was slightly elevated in comparison to the control (Table II). Among the investigated complexes, only chromium chloride significantly elevated the number of HAIRs and LAIRs. However, a simultaneous increase in the dissociation constant value (Kd) for HAIRs and LAIRs (decrease in affinity) was observed. Since the experiment was carried out on isolated membranes, fluctuations in the number of binding sites could not have been due to the effect of internalization, but rather reflected changes in the amount of insulin receptor particles accessible to insulin after treatment with chromium(III). Thus, certain Cr complexes probably masked the real number of receptors in the membranes. Receptor-insulin cooperation is complicated and its mechanisms, in spite of many studies undertaken, remain the subject of investigation. Different possibilities of bilateral binding, dimerization, hexamerization and cooperative interactions between receptors have been considered (38,42). Because of the intimate contact of the receptor with membrane lipids, each alteration in the composition or the physical state of the

lipids caused by any of the used complexes may have affected the properties of the insulin receptor. The importance of the physical state and fluidity of membranes was emphasized in a study on the influence of hypothermia on the interaction of insulin with the receptor (14). Unlike membranes, the *in vitro* model of cultured cells did not provide the possibility to verify the different effects of the chromium(III) complexes on total specific insulin binding and the kinetics of this process. This was due to the extremely low binding ability of the cells to bind the hormone (Table III); however, the cultured cells were useful for the investigation of the intracellular steps of insulin action.

Chromium(III) complexes have been identified as modulators of the insulin effect in both *in vivo* and *in vitro* studies (43,44). In the present study, we confirmed that different Cr complexes may regulate insulin signaling pathways. We found, for the first time, that chromium(III) glycinate and chromium(III) acetate complexes, in the presence of insulin, profoundly inhibited serine phosphorylation of the IRS-1 in the 3T3-L1 cell line, while chromium chloride and chromium propionate complexes did not exhibit an inhibitory effect. However, it was previously shown that the Cr propionate complex has a more potent effect on insulin secretion than on insulin sensitivity (45). Nevertheless, we found that the analyzed Cr complexes did not activate the phosphorylation of tyrosine in IRS-1 in the presence of insulin. However, chromium(III) glycinate and chromium(III) acetate to some extent increased tyrosine phosphorylation in the IRS-1 in the absence of insulin. These results indicate that the chromium glycinate and acetate complexes may play a crucial role in enhancing insulin signaling inside cells. Our results are consistent with the data obtained by Muller *et al* (46), who found that Cr complexes affect tyrosine phosphorylation in the IRS-1. However, our experiment did not confirm changes in IRS-1 phosphorylation at the tyrosine residue after treatment of the cells with the chromium(III) chloride and chromium(III) propionate complexes. On the other hand, Chen *et al* (43) found that chromium(III) chloride increased insulin-stimulated glucose transport by GLUT4 mobilization in the 3T3-L1 cell line, though regulation of glucose transport through changes in GLUT4 translocation did not involve IR, IRS-1, phosphatidylinositol 3-kinase and Akt. They also suggested that this mechanism was associated with the composition of membrane lipids, particularly with cholesterol depots.

Similar results were presented by Pattar *et al* (47), who found that chromium picolinate decreased the content of membrane cholesterol in 3T3-L1 cells. This mechanism was connected with enhanced glucose transport stimulated by insulin and increased the translocation of GLUT4 into the plasma membrane. These data indicate that Cr improves glucose transport by the modification of lipid depots and represses lipid-induced insulin resistance. On the other hand, our results showed that Cr complexes may modify the insulin signal transduction modulating process of amino acid phosphorylation in IRS-1. Moreover, it was found that Cr increases the phosphorylation of tyrosine in insulin receptor particles (48). Though these results were not confirmed in other experiments, they suggest that the mechanisms of Cr action are of high complexity. Additionally, Yang *et al* (49) reported that chromium phenylalanine increased the phosphorylation of (308) tyrosine in Akt inside insulin-stimulated adipocytes in a dose- and time-dependent manner. On the other hand, these authors did not find any influence of the Cr complex on the phosphorylation of the insulin receptor subunit (IRβ). The results presented by us and by other authors indicate that Cr action on the insulin signal cascade depends on a combination of various factors, including the amount and affinity of insulin receptor particles, the possibility of the phosphorylation of the receptor subunit and the modulation of signal proteins by changes in their phosphorylation.

Acknowledgements

This research was funded by the Ministry of Education and Science (grant no. 2 P06T 03830) between 2006 and 2009.

References

1. Chan JM, Rimm EB, Colditz GA, Stampfer MJ and Willett WC: Obesity, fat distribution and weight gain as risk factors for clinical diabetes in men. Diabetes Care 17: 961-969, 1994.
2. Reaven GM: Banting lecture 1988. Role of insulin resistance in human disease. Diabetes 37: 1595-1607, 1988.
3. Saltiel AR: New perspectives into the molecular pathogenesis and treatment of type 2 diabetes. Cell 104: 517-529, 2001.
4. Proietto J: Mechanisms of insulin resistance caused by nutrient toxicity. Hepatol Res 33: 87-91, 2005.
5. Kuryl T, Krejpcio Z, Wojciak RW, Lipko M, Debski B and Staniek H: Chromium(III) propionate and dietary fructans supplementation stimulate erythrocyte glucose uptake and beta-oxidation in lymphocytes of rats. Biol Trace Elem Res 114: 237-248, 2006.
6. Sahin K, Onderci M, Tuzcu M, *et al*: Effect of chromium on carbohydrate and lipid metabolism in a rat model of type 2 diabetes mellitus: the fat-fed, streptozotocin-treated rat. Metabolism 56: 1233-1240, 2007.
7. Shinde UA, Sharma G, Xu YJ, Dhalla NS and Goyal RK: Anti-diabetic activity and mechanism of action of chromium chloride. Exp Clin Endocrinol Diabetes 112: 248-252, 2004.
8. Virkamaki A, Ueki K and Kahn CR: Protein-protein interaction in insulin signaling and the molecular mechanisms of insulin resistance. J Clin Invest 103: 931-943, 1999.
9. Mackowiak P, Nogowski L and Nowak KW: Comparison of erythrocyte insulin receptors in different species of vertebrates. Naturwissenschaften 79: 413-415, 1992.
10. Torlinska T, Mackowiak P, Nogowski L, Nowak KW, Madry E and Perz M: Characteristics of insulin receptor binding to various rat tissues. J Physiol Pharmacol 49: 261-270, 1998.
11. Mackowiak P, Ginalska E, Nowak-Strojec E and Szkudelski T: The influence of hypo- and hyperthyreosis on insulin receptors and metabolism. Arch Physiol Biochem 107: 273-279, 1999.
12. Mackowiak P, Nogowski N and Nowak KW: High-carbohydrate and high protein diets alter liver and brain insulin receptors in rats. J Anim Feed Sci 2: 207-214, 1993.
13. Torlinska T, Mackowiak P, Nogowski L, *et al*: Age dependent changes of insulin receptors in rat tissues. J Physiol Pharmacol 51: 871-881, 2000.
14. Torlinska T, Perz M, Madry E, Hryniewiecki T, Nowak KW and Mackowiak P: Effect of hypothermia on insulin-receptor interaction in different rat tissues. Physiol Res 51: 261-266, 2002.
15. White MF: The IRS-signaling system: a network of docking proteins that mediate insulin and cytokine action. Recent Prog Horm Res 53: 119-138, 1998.
16. Wilden PA, Kahn CR, Siddle K and White MF: Insulin receptor kinase domain autophosphorylation regulates receptor enzymatic function. J Biol Chem 267: 16660-16668, 1992.
17. Myers MG Jr and White MF: Insulin signal transduction and the IRS proteins. Annu Rev Pharmacol Toxicol 36: 615-658, 1996.
18. Previs SF, Withers DJ, Ren JM, White MF and Shulman GI: Contrasting effects of IRS-1 versus IRS-2 gene disruption on carbohydrate and lipid metabolism in vivo. J Biol Chem 275: 38990-38994, 2000.

19. Withers DJ and White M: Perspective: the insulin signaling system – a common link in the pathogenesis of type 2 diabetes. Endocrinology 141: 1917-1921, 2000.
20. Carvalho CR, Brenelli SL, Silva AC, Nunes AL, Velloso LA and Saad MJ: Effect of aging on insulin receptor, insulin receptor substrate-1 and phosphatidylinositol 3-kinase in liver and muscle of rats. Endocrinology 137: 151-159, 1996.
21. Pederson TM, Kramer DL and Rondinone CM: Serine/threonine phosphorylation of IRS-1 triggers its degradation: possible regulation by tyrosine phosphorylation. Diabetes 50: 24-31, 2001.
22. Qiao LY, Goldberg JL, Russell JC and Sun XJ: Identification of enhanced serine kinase activity in insulin resistance. J Biol Chem 274: 10625-10632, 1999.
23. Saad MJ, Hartmann LG, de Carvalho DS, Galoro CA, Brenelli SL and Carvalho CR: Effect of glucagon on insulin receptor substrate-1 (IRS-1) phosphorylation and association with phosphatidylinositol 3-kinase (PI 3-kinase). FEBS Lett 370: 131-134, 1995.
24. Vincent JB: Elucidating a biological role for chromium at a molecular level. Acc Chem Res 33: 503-510, 2000.
25. Zick Y: Ser/Thr phosphorylation of IRS proteins: a molecular basis for insulin resistance. Sci STKE 2005: 4, 2005.
26. Hininger I, Benaraba R, Osman M, Faure H, Marie Roussel A and Anderson RA: Safety of trivalent chromium complexes: no evidence for DNA damage in human HaCaT keratinocytes. Free Radic Biol Med 42: 1759-1765, 2007.
27. Bagchi D, Stohs SJ, Downs BW, Bagchi M and Preuss HG: Cytotoxicity and oxidative mechanisms of different forms of chromium. Toxicology 180: 5-22, 2002.
28. Speetjensa JK, Crowderb MW, Vincent JB and Woski SA: Low-molecular-weight chromium-binding substance and biomimetic $[Cr_3O(O_2CCH_2CH_3)_6(H_2O)_3]^+$ do not cleave DNA under physiologically-relevant conditions. Polyhedron 18: 2617-2624, 1999.
29. Sun Y, Ramirez J, Woski SA and Vincent JB: The binding of trivalent chromium to low-molecular-weight chromium-binding substance (LMWCr) and the transfer of chromium from transferrin and chromium picolinate to LMWCr. J Biol Inorg Chem 5: 129-136, 2000.
30. Vincent JB: Recent advances in the nutritional biochemistry of trivalent chromium. Proc Nutr Soc 63: 41-47, 2004.
31. Clodfelder BJ, Chang C and Vincent JB: Absorption of the biomimetic chromium cation triaqua-mu3-oxo-mu-hexapropionatotrichromium(III) in rats. Biol Trace Elem Res 98: 159-169, 2004.
32. Earnshaw A and Lewis J: Chemistry of polynuclear compounds. Part VI. Magnetic properties of trimeric chromium and iron carboxylates. J Chem Soc A: 1656-1663, 1966.
33. Wieczorek D, Staniek H, Krejpcio Z, Zielinski R and Szymusiak H: Synthesis and selected physicochemical properties of novel biomimetic complexes of chromium(III). I. Complexes with acetic and lactic acids. J Hum Nutr Metab 34: 1191-1196, 2007.
34. Wieloch A, Staniek H, Szymusiak H, Krejpcio Z and Zieliński R: Tricentric complexes of chromium(III) with glycine and serine ligands. Curr Trends Commod Sci 2: 1080-1086, 2007.
35. Wieloch D, Staniek H, Krejpcio Z, Szymusiak H and Zieliński R: Synthesis and selected physicochemical properties of novel biomimetic complexes of chromium(III). II. Complexes of chromium(iii) with alanine and glycine. J Hum Nutr Metab 34, 2007.
36. Havrankova J, Roth J and Brownstein M: Insulin receptors are widely distributed in the central nervous system of the rat. Nature 272: 827-829, 1978.
37. Munson PJ and Rodbard D: Ligand: a versatile computerized approach for characterization of ligand-binding systems. Anal Biochem 107: 220-239, 1980.
38. Gammeltoft S: Insulin receptors: binding kinetics and structure-function relationship of insulin. Physiol Rev 64: 1321-1378, 1984.
39. Shindea UA, Sharma G, Xu YJ, Dhalla NS and Goyal RK: Insulin sensitising action of chromium picolinate in various experimental models of diabetes mellitus. J Trace Elem Med Biol 18: 23-32, 2004.
40. Broadhurst CL and Domenico P: Clinical studies on chromium picolinate supplementation in diabetes mellitus – a review. Diabetes Technol Ther 8: 677-687, 2006.
41. Martin J, Wang ZQ, Zhang XH, *et al*: Chromium picolinate supplementation attenuates body weight gain and increases insulin sensitivity in subjects with type 2 diabetes. Diabetes Care 29: 1826-1832, 2006.
42. Ward C, Lawrence M, Streltsov V, *et al*: Structural insights into ligand-induced activation of the insulin receptor. Acta Physiol 192: 3-9, 2008.
43. Chen G, Liu P, Pattar GR, *et al*: Chromium activates glucose transporter 4 trafficking and enhances insulin-stimulated glucose transport in 3T3-L1 adipocytes via a cholesterol-dependent mechanism. Mol Endocrinol 20: 857-870, 2006.
44. Wang ZQ, Zhang XH, Russell JC, Hulver M and Cefalu WT: Chromium picolinate enhances skeletal muscle cellular insulin signaling in vivo in obese, insulin-resistant JCR:LA-cp rats. J Nutr 136: 415-420, 2006.
45. Bunting LD, Tarifa TA, Crochet BT, Fernandez JM, Depew CL and Lovejoy JC: Effects of dietary inclusion of chromium propionate and calcium propionate on glucose disposal and gastrointestinal development in dairy calves. J Dairy Sci 83: 2491-2498, 2000.
46. Muller G, Jung C, Wied S, Welte S, Jordan H and Frick W: Redistribution of glycolipid raft domain components induces insulin-mimetic signaling in rat adipocytes. Mol Cell Biol 21: 4553-4567, 2001.
47. Pattar GR, Tackett L, Liu P and Elmendorf JS: Chromium picolinate positively influences the glucose transporter system via affecting cholesterol homeostasis in adipocytes cultured under hyperglycemic diabetic conditions. Mutat Res 610: 93-100, 2006.
48. Brautigan DL, Kruszewski A and Wang H: Chromium and vanadate combination increases insulin-induced glucose uptake by 3T3-L1 adipocytes. Biochem Biophys Res Commun 347: 769-773, 2006.
49. Yang X, Palanichamy K, Ontko AC, *et al*: A newly synthetic chromium complex – chromium(phenylalanine)3 improves insulin responsiveness and reduces whole body glucose tolerance. FEBS Lett 579: 1458-1464, 2005.