The chromium(III)-ions and diabetes

Ph.D. thesis

Zsuzsanna Keszthelyi M.D.

University of Pécs Medical School
Ist Department of Medicine

Program leader: Gyula Mózsik M.D. D.Sc.

Pécs

2005.

INTRODUCTION

Nowadays the prevalence of the type 2 diabetes mellitus is still growing. Although the occurrence of the insulin resistance is quite often in the whole population, diabetes not always develops because for a time the compensating mechanism avoids it. In a frequent variation of type-2 diabetes the disease is not the result of an alteration in the insulin receptor or the glucose transporter, but a genetically determined defect of the postreceptorial intracellular signaling mechanism plays a role in its occurrence. There have been investigations for decades to find out more about the role of chromium (III) ions in glucose metabolism and in the prevention of type-2 diabetes. It has also been investigated if chromium substitution can prevent or treat those forms of diabetes where chromium deficiency is suspected to be in the background of the disorder.

Since the recognition of the role of chromium(III)ions in glucose tolerance in 1959, numerous studies have dealt with the role of nutritional chromium in glucose and lipid metabolism. According to our present knowledge chromium exerts its effect through augmenting insulin's effect, although the mechanism of action is not yet fully known. The effect of chromium is supposed to manifest through the blockade of the insulinase enzyme and the elevation of effective insulin receptors. Insufficient chromium intake may lead to impaired glucose tolerance, diabetes mellitus, hyperlipidaemia, and as a consequence to the appearance of cardiovascular diseases. According to the results of several studies, nutritional chromium intake in western societies is suboptimal (daily requirement of chromium(III) ions is 50-200 μg .

The brain plays an essential role in monitoring and regulating the energy balance of the body. Carbohydrate and adipose stores are monitored through neural and metabolic signals from the periphery. Carbohydrate metabolism is especially important for the brain as it uses glucose levels are monitored by both direct and indirect ways.

The ventromedial hypothalamus (VMH) has been recognised as "satiety center " for a long time. Lesions in the VMH disrupt production and reception of satiety signals that causes Hyperphagia and abnormal body weight gain. VMH lesions are frequently accompanied by increased insulin secretion and elevated levels of FFA, as well as increased gastric and acid secretion. Oomura et al. and Anand et al. have identified neurons within the areas of the LHA and the VMH that altered their firing rates when plasma glucose levels elevated. Oomura et al. later showed that directly applied glucose altered the firing rate of certain neurons that of

the so-called “glucose monitoring (GM) neurons. They defined “glucose-receptor” (GR) neurons that increased their firing rates when nearby glucose levels rose, and “glucose-sensitive”(GS) neurons that decreased their firing rate under the same circumstances. In the LHA, VMH, as well as in the solitary tract 20-40 % of all neurons show such properties.

Previous works show that the insulin-producing β cells of the pancreas and the glucose monitoring neurons in the forebrain shave common features: 1. The same ATP-sensitive K-channels cab be found in both kinds of cells, 2. the GLUT-2 glucose transporter is present in both type of cells, 3. the same sulphanylurea-receptors. It is known that streptozocin (STZ) selectively destroys the insulin-producing β-cells of the pancreas after getting into these cells through the GLUT-2 “receptor”. Karádi and al. showed that direct microinjections of STZ to the VMH cause symptoms very similar to those of type-2 diabetes mellitus. In extracellular single neuron activity recording studies. STZ proved to be specifically toxic to GM neurons. These findings indicate that STZ exclusively destroys the glucose monitoring neurons of this area, which causes the observed aberrations in carbohydrate metabolism, that the similar to those seen after classical lesions of the VMH.

Clinical studies have reported that longstanding chromium deficiency causes impaired glucose tolerance or diabetes mellitus. It was also proved that with age, chromium concentrations decrease in the central nervous system.

Selenium, an essential nutrient, exerts its action as an antioxidant via its incorporation into glutathione peroxidase, an enzyme found also in erythrocytes. The activity of glutathione peroxidase in most tissues is highly sensitive to dietary levels of selenium, which is bound to the active site as a selenocysteine and serves as the redox center in catalysis. Deficiency of selenium is accompanied by a decrease in the activity of glutathione peroxidase. Selenium is the cofactor of phosphoglucomutase, a key enzyme in glycolysis and glucose synthesis. Selenium has been shown to mediate insulin-like actions both in vivo and in vitro.

Intracellular magnesium is involved in adenosine triphosphate phosphorylation and a co-factor for the adenylate cyclase enzyme. Intracellular magnesium may enchance insulin action through insulin receptors, the insulin-mediated glucose uptake and insulin-stimulated phosphorylation of tyrosin kinase on insulin receptor.

Presence of magnesium is important for normal insulin action and red blood cell viscosity, while magnesium deficiency results in impaired insulin secretion and reduced tissue sensitivity to insulin. The erythrocytes have no insulin receptor.

AIM

Chromium (III) has an important role in the regulation of insulin action influencing carbohydrate and lipid metabolism.

The aim of this study was to clarify the effect of chromium and selenium on glucose metabolism at in vitro circumstances. To investigate the role of the presence of these elements on net glucose consumption by a newly developed surviving erythrocyte suspension. To examine the effect of chromium and selenium on glucose metabolism of insulin receptor absent cells.

In my present study we examined a hypothetised protective capacity of Cr(III) ions on STZ treated male Wistar rats. A single bilateral STZ microinjection into the VMH elicited pathological elevation of blood glucose levels in acute as well as in chronic glucose tolerance tests.

The aim of our present investigation is to test the role of chromium (III) compounds in glucose metabolism that are known from literature. The author examined the effect of oral chromium supplementation on the antidiabetic treatment. Chromium supplementation was applied for 6 months. Knowing contemporary dietary habits, the prevention and the treatment of chromium deficiency requires the substitution of artificial chromium containing agents, as well as dietary modifications. Administration of such agents in diabetes mellitus is accepted, although their efficiency is still debated. In our present study we examined the efficiency of a novel chromium containing agent in diabetic patients prepared in our clinic.

Chromium(III) containing agents should possess the following main properties:

-It should contain chromium (III) in the form of a well defined compound with an adequate complex stability constant and bioavailability value

-The amount of chromium (50 μg/g) should be safely to dose during preparation as well as during intake

-Vehiculum should be well definable

-The properties of the vehiculum should be adequate for the treatment of the primary disease

-The vehiculum should not have pharmacological properties

-It should possibly improve, but by no means worsen the bioavailability of the effective agent (chromium (III)).

-The presentation of the drug should minimize insecurities that are due to drug-adherence.

According to my knowledge there is no such agent available at the present in commerce so we prepared a special chromium(III) containing agent based on our patent "Procedure for the preparation of fiber products" and "Procedure for preparation of fiber product containing biologically active chromium complex". The product was prepared and dosed under constant quality control in our laboratory.

METHODS

Red blood cell preparation:Approximately 300 ml whole blood was obtained from each patient with polyglobulia as part of therapeutic blood taking. RBC samples were heparinised then centrifuged (1000x g, 10 minutes, 0-4 ^{0}C) immediately.

Chemiclas used in the experiments were chromium picolinate complex (prepared according to Evans and Poutschnik method, selenium, magnesium and isoosmotic glucose solutions (Reanal Ltd., Budapest, Hungary) and insulin (40 IU/ml, Novo Nordisk, Actrapid HMge). Sterile 0.9% saline was used for vehicle.

Incubations, Protocols: The first solution contained chromium-picolinate in 80 μM concentration solved in physiological saline. The second solution contained H_2SeO_3 in 16 μM concentration, solved in serum. The third solution contained $MgCl_2$ in 8 mM concentration, solved in 141 mM saline solution. The applied magnesium and H_2SeO_3 were obtained from Reanal (Budapest, Hungary), and were pro analyze quality. The fourth solution was a 1600 IU/ml insulin (Actrapid HMge 40 E/ml, Novo Nordisk) solution, solved in physiological saline. The fifth solution was an approximately isoosmotic solution used for the preparation of incubation mediums. Contained 10mM glucose solved in 145 mM saline solution, which contained 4 mM NaH_2PO_4P-Na_2HPO_4, pH was 7,38. The sixth solution was a mixture of physiological saline and serum. Concentration of the serum was 84% (v/v) and that of the physiological saline solution was 16 % (v/v).We used 18 samples with different composition from the previously described solutions. Hematocrite in the test tubes was 0.5 l/l. Osmolarity of the samples was in physiological range.The tubes were closed, their contents homogenized, then they were put into a 310K thermostat for 180 minutes. During incubation time the contents of the tubes were homogenized by carefully shaking at the same intervals (t=15 minutes). Sampling followed in the 0^{th}, 60^{th}, 120^{th} and 180^{th} minutes. At each sampling glucose and insulin levels in the supernatant were tested. Increase of hemoglobin level in samples indicated hemolysis. Na and K ion concentrations were determined by flame photometry. Glucose concentration of the supernatant was measured with spectrophotometry

using Antron-reagent. Insulin level in the supernatant was measured with a ^{125} I RIA kit (IZINTA, Hungary). Hemoglobin concentrations were measured via spectrophotometry using benzidine reagent.

Animals: adult, male Wistar rats weighing (250-270 g at the start of experiments were used in this study. Surgery was performed under ketamine general anesthesia (X mg/100 g). Skin, muscle and connective tissue were removed from the skull. Under microscopic control a hole 2-3 mm in diameter was made in the the skull above the VMH to reach the level of the durea mater. A leading canule was placed on the dura meter above the area of the VMH and it was fixed with dentist's acrilate to the skull.After a week-long recovery period the administering canules were placed into the leading canules. The animals were divided into 3 groups. Materials were delivered into the VMH through the administering canule with the help of a Hamilton-microsyringe and a microinjection pump. 1 μl of each material was administered on both sides. Before pulling out of the administering canule, we waited 1 minute for the materials to diffuse from the place of administration. The rats were divided into 3 groups. One group was given a chromium-picolinate pre-treatment (2 mg/ml, pH 5), then 75 minutes later STZ (dissolved in physiological saline, 10 mg/ml, pH 6,5). A second group was given only STZ and a third group sham-operated group was given the same amount of saline. Procedures: an acute OGTT was made 15 minutes after the administration of the microinjections. After taking the animals' fasting blood sugar level, OGTT was performed through a catheter placed into the stomach through the mouth. Blood sugar levels were measured 9, 18, 30, 60 and 120 minutes after the administration of 0,5 mg/ml/100 g D-glucose solution.

After 15 hours fasting the animals got 6 NE/kg insulin intraperitoneally. Their food uptake was measured 2, 4 and 24 hours after the injection. Blood sugars were also measured at the same time. Plasma insulin and leptin levels were determined with the help of radioimmunoassey kits (Linco Co., USA). Histological studies were performed to determine if the administering canules were positioned correctly.

In the study was performed on diabetic patients who have been treated in our diabetology outpatient clinic for a longer time. The time frame of the study was defined in 6 months according to literature. We included 20, type 2 diabetic patients (age: 40-65) in the study whose disease have been treated for a longer time (1-10 years) with oral antidiabetics, as well as one newly recognized diabetic patient. 10 randomly chosen patients treated with oral antidiabetics as well as the formerly not treated patient received oral chromium supplementation with the developed product. The other 10 patients included into the study (

mean age: 40-65) served as control group in the study. During the study these patients did not receive chromium therapy.

The patients participated in the study voluntarily. The tests were performed based on a protocol and on the permission of the Local Committee for Research Ethics. During our study we did not observe any adverse effects in the group receiving the examined product. 2 patients from the group that received chromium supplementation refused further contribution. No side-effects were observed in their case either. In our study we determined vital parameters, changes in body weight and the following clinical chemical parameters: fasting blood sugar, fructoseamine, hemoglobin A_{1C}, serum cholesterol, serum HDL cholesterol and their ratio. During evaluation we examined changes in these parameters before and after the beginning of treatment in the fraction of time.

Main parameters of the applied product:Vehiculum: nutritional rough fiber, water binding capacity 10g/g fiber, oil binding capacity 5 g/g fiber , water soluble part: according to patent. Active ingredient: chromium $^{3+}$ picolinate , chromium content 50 µg chromium metal/unit.

RESULTS:

The vital function had been sustained with simple methods on an approximately constant activity. For these reasons this red blood cell suspension model has been chosen because it is available in big quantities, sustaining its vital functions is simple, membrane permeability processes (chromium (III)-ions, insulin effect) are easy to follow while stability and integrity of the system are easy to control. Glucose consumption is suitably intensive and time related changes in the stability of the insulin molecule can be measured easily and sensitively, and are easy to follow.Cell degradation:Hemoglobin concentrations were below 15-20 mg% during the whole experiment. No changes regarding the composition of the incubation mediums were observed.In the samples taken in the 180th minute potassium levels were not higher than 0.4-0.5 mM. Considering the proportion of volumes of the cell suspension and the incubation medium (1:1) and the fact that potassium concentration inside the cell is as high as 70-90 mM, and presuming that all the measured potassium was a result of the disintegration of cell membranes, we can calculate the proportion of damaged cells. Developing this thought, if we take the lower limit of potassium concentration inside the cell, and the upper limit of potassium concentration in the incubation medium (as the least favorable extremes), we can see that less than 0.7% of the cells disintegrated. In reality, much less cells were destroyed, as

most potassium got into the intercellular space from cells with intact membranes via passive diffusion, regarding the high concentration gradient and the long incubation time (180 min). This presumption is also supported by the conclusions that can be drawn from hemoglobin concentrations. Making the calculation where average hemoglobin concentration in red blood cells is 261g/l (blood hemoglobin concentration: 145 g/l, HTC: 0.5 L/L), that means 36.3 mg%, the proportion of disintegrated cells would be 0.14%. K^+ and hemoglobin concentrations in the incubation medium:Measuring potassium and hemoglobin concentration in the supernatant enabled us to follow changes in the integrity of red blood cells in the samples during the incubation process. We presumed that partial or total disintegration of the cell membranes would be easy to follow through changes in the potassium and hemoglobin levels of the incubation medium. The incubation medium contained both hemoglobin and potassium in immeasurable amounts at the point when the different components were measured into the samples. Regarding that potassium and hemoglobin levels are high in the intracellular space, and there are simple and sensitive methods to detect them, the applied methods seemed suitable to answer the raised questions.

Glucose consumption:The quantity of glucose was measured in the medium at 60, 120 and 180 min after start of experiments. Consumption data were expressed as the fraction of total applied glucose at zero time. The figure summaries the result of examination of the different incubation mediums, shows changes in glucose concentrations in percentages in the different samples compared to the controls, at the time of the different samplings. Chromium with or without selenium in the medium induced a higher level of glucose consumption ($p<0.05$) at the 120^{th} and 180^{th} min. Samples containing insulin, chromium and magnesium with selenium increased glucose consumption ($p<0.05$) at the 180^{th} min, without selenium these samples reached a statistically significant decrease only at the 120^{th} and 180^{th} min. There was no difference from respective controls at the other time points. Presence of magnesium in itself in the medium did not increase glucose consumption, however magnesium and selenium induced an increased consumption level at the 180^{th} min. Insulin with or without selenium did not change glucose consumption level from the samples compared to respective control group. Insulin and magnesium together filed to change glucose consumption, but adding selenium to the medium resulted a statistical difference ($p<0.05$) at the 180^{th} min. Insulin and chromium with or without selenium in the medium resulted no changes in the glucose consumption level during the experimental period.Insulin levels:Samples not added with insulin could not be compared to controls while not having a standard insulin concentration at the start of experiments. Samples added with insulin alone were accepted as control group.

The insulin level had a time related decrease in the control samples containing insulin alone, and the reduction rate of insulin level got decreased when selenium was present ($p<0.05$). Selenium decreased ($p<0.05$) insulin elimination level at the 60^{th}, 120^{th} and 180^{th} min. Magnesium with or without selenium induced a similar inhibition pattern to effect of selenium alone. Chromium decreased insulin elimination level ($p<0.05$), and this effect of chromium was potentiated by presence of selenium in the samples. Presence of chromium and magnesium together in the incubation medium causes a prolonged decrease (in the 60^{th}, 120^{th} and 180^{th} min) in insulin elimination. Chromium, magnesium with selenium together cause a similar insulin elimination pattern to selenium itself.

There was significant difference between and the both STZ and chromium treated groups in acute OGTT tests. The group that was administered STZ only showed a pathological rise in blood sugar levels. The group that was chromium pre-treated before getting the STZ didn't show significant differences in their blood sugar levels compared to the shame-operated group. Chromium pretreatment proved to be effective against the acute effects of STZ. In chronic OGTT a prolonged curve appeared in the STZ treated group.

According to statistical analysis blood sugar levels ($r=0,041$, $p<0,661$), total cholesterol levels ($r=0,669$, $p<0,02$) and $HgbA_{1c}$ levels ($r=0,518$, $p<0,06$) changed significantly. The other results did not show significant changes although their value decreased during the examined 6 months. Due to their results the authors presume that chromium (III) compounds may be effective in the treatment of patients with decreased glucose tolerance or type-2 diabetes mellitus as a supplement to their therapy.

DISCUSSION:

We have introduced a novel in vitro method to investigate chromium, selenium and magnesium effect on glucose consumption and insulin degradation in human red blood cells, a cell population not having insulin receptor.

Control samples containing RBCs suspended in saline did have a linear glucose consumption curve in time. Chromium and selenium added into the medium increased glucose level decrease at the 120^{th} and 180^{th} min of incubation, however chromium or selenium had no effect themselves. Insulin, chromium, magnesium and selenium together induced a delayed

higher glucose consumption starting at the 180th min. Addition of chromium with insulin or magnesium did not change glucose elimination from the medium.

In the other part of the experiment a standard insulin concentration was set in incubation media and the decrease of insulin level was detected in time. In control groups insulin decrease was exponential. Selenium, magnesium, chromium themselves and chromium with magnesium decreased insulin level decrease in the first 60 min, however in the 120th and 180th min there was no the difference from controls. Selenium with magnesium in the medium did not cause any change in insulin levels compared to controls. The most effective combination in our set up was chromium, magnesium and selenium together inducing a prolonged –both 60th, 120th and 180th min- decrease in insulin level.

From our results we conclude that presence of chromium and selenium together induce an increased glucose consumption, and this process does not need presence of insulin in cells without insulin receptor. Selenium maybe stabilize insulin in itself, however magnesium or chromium can decrease this effect. Chromium and magnesium in themselves or together also can stabilize insulin molecule.

Selenium is the cofactor of phosphoglucomutase, a key enzyme in glycolysis and glucose synthesis. It has been shown to mediate insulin-like actions both in vivo and in vitro. Taking together with our results these suggest that selenium increase glycolysis in an insulin receptor free cell population.

It has been shown that chromium coupling with insulin enhances insulin activity. In our model insulin degradation was reduced by chromium suggesting that increase of insulin activity is due by a stabilizing effect of the element.

Intracellular magnesium is involved in adenosine triphosphate phosphorylation and a co-factor for the adenylate cyclase enzyme. Intracellular magnesium may enchance insulin action through insulin receptors, the insulin-mediated glucose uptake and insulin-stimulated phosphorylation of tyrosin kinase on insulin receptor.

Magnesium is an essential cofactor for a multitude of enzymatic reactions that are important for the generation of energy from ATP and for physiologic processes. Magnesium is essential for normal cell function. In our experiments magnesium had a stabilizing effect on insulin and this can be an important factor in insulin action. The chromium containing substances may play an important role in physiological and patophysiological processes of glucose metabolism, i.e. development and maybe in the therapy of diabetes mellitus disease.

According to the previous results, by the effect of the microinjection of VMH STZ, both in acute and chronic pathology high blood sugar values came up tested by oral glycose tolerancy.

The STZ used centrally also caused the increase of neutrition intake due to the ip. insulin. In the group where we used pretherapy with chromium, these malfunctions were not present. We could not show any significant alteration in the level of insulin or leptin in any of the groups.

In our experiment the pretherapic use of chromium stopped the development of the symptoms of diabetes that can be generated by the treatment of streptozotocin. By the observations of our earlier experiments, the STZ added into VMH kills the gylcose monitoring neurocells which can be located here. Therefore we presume that the local changes of the chromium level of the brain migh effect the mechanism of this glycose receptor neurons.

Karádi and his co-workers made it clear by their experiments that the STZ microinjection added to VMH causes disfunctions in adaptations in the treated animals, after the intake of a higher portion of glycose. During the animal tests abnormal glycose tolerancy developped, just like in case of human diabetes mellitus. The result relates other VMH lesion data in medical records, where after electric or chemical destruction hyperglycemia was observed. There is a good reason to presume that the changes of glycose tolerancy are due to the death of the GR cells of VHM, since in the pancreas, where the harmful effect of the beta cells of STZ could have caused similar alterations, we did not find any signs of patomorphological death of the cells.

By analyzing the argumental interrelations the following things can be expected:

The plasma insulin significantly dicreases $\rightarrow$ type 1 of diabetes mellitus

The plasma insulin significantly increases $\rightarrow$ hyperinsulinemia,
insulin-resistency,
diabetes mellitus,
metabolic X-syndrome.

Further more, if the leptin level of the plasma increases we might also think of the growth of the total fatstocking. Suga and his co-researchers found in their VMH lesion experiment that the level of leptin of the plasma was six times higher two weeks after the operation. This level continued to increase even after 14 weeks.

Knowing the fact that by aging there is a reduced chromium quantity in almost every tissue, including the brain tissues as well, we can make the following conclusions:

- It is probable that the local changes of the level of chromium in the CNS effect the functioning of the glycose monitoring neurocells.
- The mechanisms that are behind of the role of the chromium in the central neurosystem are not thoroughly known, the investigation needs further examinations.
- The absolute or relative chromium absence interfere in the pathology of diabetes. It can occur, that the decrease of chromium content of the CNS with aging might correspond with the malfunctions of the central regualion of the carbohydrate metabolism.
- One can presume that several patients having type 2 of diabetes mellitus the absence of chromium might have a role in the development of the desease.

The further study of the topic might bring in important results in the possible prevention and therapy of diabetes since we can assume that numerous patients with the type 2 of diabetes mellitus the absence of chromium might have a role in the development of the desease.

I observed the elevation of efficacy of treatment in every case of antidiabetic therapy substituted with oral chromium therapy (2x50 μg daily per os). This could be observed both in the case of insulin treatment and the case of oral antidiabetics.

Although hemoglobin A1c values did not show statistically significant changes, a decreasing tendency could be observed in the group of patients receiving chromium substitution. Lack of statistical significance may have been due to the low number of cases It is known that this parameter respects the average glucose levels of 4-5 weeks previous to the blood draw, so it seems possible that chromium(III) supplementation may durably decrease glucose levels.

Among the examined parameters total cholesterol, which elevates the risk of arteriosclerosis and cardiovascular events in the diabetic population, evidently decreased significantly. A positive effect may be observed in the change of total cholesterol/HDL cholesterol ratio as well. As total cholesterol decreased significantly, and the proportion of HDL cholesterol within the cholesterol fraction augmented, therefore LDL cholesterol fraction which has a relevant atherogen potential decreased both absolutely and relatively.

Based on our results the use of chromium as supplementation is important in diabetics if the therapeutic efficacy of previously administered antidiabetics decreases. An explanation of this phenomenon might be the elevation of the sensitivity of insulin receptors. It is likely that biologically active chromium (III) compounds exert a sinergising effect on insulin receptors.

In early type 2 diabetes (freshly recognized, low elevation of blood glucose) it is reasonable to start chromium substitution even before the beginning of oral antidiabetic therapy and further therapeutic decisions should be made according to its efficacy.

Based on literature and our own examinations it can be stated that chromium(III) compounds are effective in the treatment of patients with impaired glucose tolerance and type 2 diabetes mellitus. We emphasize that -disregarding special cases-chromium therapy is proposed to be used not instead of usual antidiabetic therapy but as its substitution.