SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The effect of nickel on homocysteine metabolism in patients with end-stage renal disease on hemodialysis and *in vitro* in peripheral mononuclear cells

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The Examination takes place at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen
September 20, 2016, 11:00

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Introduction

Homocysteine is a sulfur-containing amino acid derived from methionine. Methionine is converted to S-adenosylmethionine (SAM), which functions as a methyl donor in numerous methylation reactions. Upon transfer of its methyl group, the resulting S-adenosyl homocysteine (SAH) becomes available for hydrolysis; SAH hydrolysis is the only source of homocysteine in mammals. All mammal cells can remethylate homocysteine in a reaction catalysed by methionine synthase, a reaction which closes the methionine-homocysteine cycle and connects it to the folate cycle. Reaction velocities are affected not just by substrate and product concentrations but also by the concentrations of metabolites in distant parts of the reaction network, allowing proper methionine regeneration. Homocysteine leaves the methionine cycle via the transsulfuration pathway in a reaction catalysed by the cystathionine-β-synthase (CBS). CBS activity dominates when methionine and SAM statuses are adequate, and it is enhanced by oxidative stress. Hydrolysis of cystathionine is the only reaction that results in *de novo* cysteine synthesis. Cysteine concentration is a rate-limiting factor in glutathione synthesis. Glutathione has an important role in maintaining cellular redox homeostasis.

Impaired homocysteine metabolism results in homocysteine accumulation in the plasma, a phenomenon called hyperhomocysteinemia. Plasma total homocysteine level is influenced by genetic and environmental factors. Several inheritable enzyme defects can cause hyperhomocysteinemia. Cofactor deficiencies as folate and vitamins B_6 and B_{12} can elevate plasma homocysteine level; this effect is dependent on methionine supply, the major source being protein in the diet. Plasma

homocysteine levels are elevated in chronic kidney failure from the early stage of disease and are closely related to the decrease of the glomerular filtration rate. Reduced excretion of homocysteine and intemediers of homocysteine metabolism (SAM, SAH), decreased renal metabolism of homocysteine, unfavourable effects of uremic toxins on enzymes, as well as insufficient vitamin status may contribute to the hyperhomocysteinemia in chronic kidney disease patients.

Elevated plasma level of homocysteine is an independent risk factor for atherosclerosis and thrombosis. Several mechanisms have been proposed for the homocysteine induced cardiovascular disease including altered lipid metabolism, modulation of extracellular matrix protein expression, inflammatory response and oxidative stress. Homocysteine inhibits the endothelial nitrogen-monoxide synthase enzyme and decreases the expression and activity of certain antioxidant enzymes resulting in enhanced oxidative stress. Thus, nitrogen-monoxide synthesis decreases and inactivation of nitrogen-monoxide by free radicals increases, resulting in impaired endothelium-dependent vasodilatation. Homocysteine enhances procoagulant activity, since it increases the cellular tissue factor activity, while inhibits the expression of thrombomodulin, activation of protein C and inactivation of factor Va by activated protein C. Homocysteine stimulates the expression of major chemokines in leukocyte trafficking (monocyte chemotactic protein 1 and interleukin-8), creating favourable conditions for the initiation and progression of atherosclerosis. Elevated homocysteine level enhances homocysteine-thiolactone generation; homocysteine-thiolactone induces apoptotic cell death, and is more toxic to cultured cells than homocysteine itself. Homocysteine-thiolactone bonds to protein lysine residues (N-homocysteinylation), which enhances their

susceptibility to oxidation, formation of intramolecular disulfide bonds and aggregates. Thus, N-homocysteinylation leads to altered protein function and may trigger autoimmune response. Homocysteine accumulation inhibits SAH hydrolysis, leads to elevated SAH concentration, which decreases the activity of SAM-dependent transmethylases resulting in hypomethylation. Altered methylation is a risk factor for cancer. Elevated homocysteine level has a role in the development of neural tube defects and certain neurodegenerative diseases.

Several studies have confirmed the effectiveness of vitamin supplementations, including folate, vitamins B_6 and B_{12}, in reducing hyperhomocysteinaemia. In chronic renal disease patients, hyperhomocysteinemia can be reduced by vitamin supplementation in most cases. Homocysteine-lowering treatment is effective in reducing cardiovascular risk in severe hyperhomocysteinemic patients. However, lowering plasma homocysteine level utilizing vitamin therapy does not decrease cardiovascular risk in subjects with mild hyperhomocysteinemia, either in the general population or in patients with end-stage renal failure or in kidney transplant recipients. In attempts to modify homocysteine metabolism, it has been proven that nickel and cobalt decrease homocysteine levels in animals with vitamin B_{12} deficiency. Homocysteine has been found to decrease in copper deficient rats.

Nickel, cobalt and copper belong to the transition metals. Under physiological conditions, most transition metals can exist in multiple valence states and thus can participate in single electron transfer reactions as components of enzymes or in formation of reactive oxygen intermediers (ROI). So far, no essential function of nickel was found in higher eukaryotes, but in animals nickel deprivation was reported to have adverse

effects on growth, reproduction and liver function. Cobalt, as a component of vitamin B_{12}, is required for function of several enzymes. Copper is essential for normal haematopoiesis, and several enzymes are known to be copper-dependent. Nickel, cobalt and copper are toxic in excess, and generate oxidative stress. Reaction of Ni(II)-thiol complexes and molecular oxygen, and/or lipid hydroperoxides, could play a major role in the free radical generation by nickel. Nickel exhibits immunomodulatory and immunotoxic effects, which explain the nickel induced allergic dermatitis. The mechanisms of cobalt and nickel carcinogenicity involve the direct interaction with DNA and nuclear proteins, and inhibition of DNA repair. Nickel may interfere with iron homeostasis at both the levels of extracellular transport and intracellular utilization by preventing iron from entering the cell, and by competing for iron sites on enzymes.

Objectives

- Our objective was to assess nickel, cobalt and copper status as well as their correlations with plasma homocysteine, folate and vitamin B_{12} levels, parameters of iron metabolism and markers of malnutrition and inflammation in patients with end-stage renal disease on haemodialysis and in healthy subjects.

- We aimed to study the homocysteine metabolism *in vitro* in a cellular model, which possesses both remethylation and transsulfuration, and is suitable for examination of individual differences without invasive techniques. We hypothesized that the peripheral blood mononuclear cells may be proper candidates for carrying out these tests.

- Using the established above model system, we aimed to study the effects of elementscorrelated with homocysteine level on homocysteine metabolism at concentrations found in renal patients and healthy subjects.

Patients and methods

Patients on haemodialysis and healthy subjects

One hundred and twenty two patients with end-stage renal disease were enrolled in the study (57 female and 65 male, mean age: 60.1±12.3 years. Their haemodialysis treatments were managed according to the professional standards. 50 healthy individuals were investigated as controls (31 female and 19 male, mean age: 37.2±10.1 years. None of them had any known diseases including hypertension, metabolic abnormalities, acute infections, liver and kidney malfunctions. All healthy subjects had a body mass index in the normal range (<25 kg/m^2). The study protocol was approved by the Ethics Committee of the University of Debrecen. All participants provided written informed consent. Blood was taken after an overnight fast and, in case of haemodialysis patients, before dialysis.

Total protein, albumin, haemoglobin, C reactive protein (CRP), iron, ferritin levels, transferrin saturation, vitamin B_{12} and folate levels were determined using standard laboratory methods in the Institute of Laboratory Medicine, University of Debrecen. Serum interleukin-6 (IL-6) concentrations were measured using ELISA method. Total homocysteine concentrations in plasma were determined by high pressure liquid chromatography (HPLC). Trace elements were measured by inductively coupled plasma mass spectrometry (ICP-MS).

In vitro experiments

Peripheral blood mononuclear cells were separated from heparinized blood of healthy volunteers by density gradient centrifugation.

Cells ($1x10^6$ cells/mL) were suspended in RPMI 1640 medium, placed into 24-well plates, and incubated in absence or presence of nickel-chloride, cobalt-chloride or copper-sulfate. In certain tests L-methionine, L-cysteine, L-homocysteine or reduced glutathione were added. L-homocysteine was prepared from L-homocysteine-thiolactone by alkaline hydrolysis. Cells were incubated for 24, 48 or 72 hours in a humidified CO_2 (5%) incubator at 37°C. For stimulation, Concanavalin A or Phytohemagglutinin was added to the medium supplemented with 10% heat inactivated fetal bovine serum.

Cell viability was measured by determination of MTT incorporation. To measure the apoptosis inducing effect of the elements, interleukin-2 (IL-2), tumor necrosis factor related apoptosis inducing ligand (TRAIL) and Fas ligand (FasL) were measured by ELISA technique.

Intracellular homocysteine, glutathione, SAH, SAM and released homocysteine in the supernatant were measured by HPLC. For thiols and for SAH and SAM, fluorescent and UV detectors were used, respectively.

Methionine synthase activity was determined in cell lysate by measuring the conversion of added methyl-tetrahydrofolate to tetrahydrofolate per unit time using spectrophotometer. Cystathionine-β-synthase activity was determined in cell lysate by measuring the added L-homocysteine consumption per unit time using Ellman's reagent. Protein contents of cell suspensions were determined by the method of Lowry.

Incorporation of methyl groups to DNA and lipids was measured using L-[methyl-^{14}C]-methionine. Genomic DNA methylation was measured by ELISA. ROS generation was assessed using a fluorescent probe, 2',7'-dichlorofluorescin diacetate, which was known to hydrolyse, and then to be oxidized by ROS in the cells to form a fluorescent product.

Cystathionine-β-synthase expression was measured by quantitative polymerase chain reaction (qPCR) and Western blot analysis.

Statistical analysis

Statistical analysis was performed using STATISTICA 7 software (Statsoft, Inc.). The distribution of continuous variables was checked by the Kolmogorov–Smirnov test. Correlation analysis was performed to study the relationship between continuous variables. Student's t-test or Mann-Whitney U test were used to compare continuous variables between two groups. Analysis of variance (ANOVA) followed by Newman-Keuls post hoc test and paired t-test were performed to analyse the results of *in vitro* studies. The level of statistical significance was set at $p < 0.05$. Laboratory parameters are shown as medians in the Results and discussion section.

Results and discussion

Serum trace elements, homocysteine status, parameters of iron metabolism and markers of malnutrition and inflammation

Folate or vitamin B_{12} deficiency was not found in the study population of renal patients on haemodialysis, obviously due to vitamin supplementation. Serum folate and vitamin B_{12} levels were inversely correlated with total homocysteine concentrations ($r = -0.30$, $p < 0.01$ and $r = -0.35$, $p < 0.001$ respectively), indicating effectiveness of vitamin supplementation, but mild hyperhomocysteinemia was common in patients (20.7 μmol/L) despite high vitamin levels. The serum levels of chronic inflammation markers CRP (4.8 vs 1.1 mg/L, $p < 0.01$) and IL-6 (7.7 vs 2.2 ng/mL, $p = 0.001$) were higher, while albumin (38 vs 48 g/L, $p = 0.0001$)

and total protein (69 vs 77 g/L, p = 0.0001) levels were lower in haemodialysis patients than in healthy subjects. In haemodialysis patients, albumin level have recently been reconsidered as a marker of general illness rather than malnutrition; conditions with albumin lowering effect are chronic inflammation, infections, peripheral vascular diseases, uraemia and acidosis. Decreased serum albumin and total protein levels predict poor prognosis in end-stage renal disease patients. Markedly elevated serum ferritin levels (400 vs 37 μg/L) were found in haemodialysis patients as a marker of inflammation and functional iron deficiency. As a consequence of iron deficiency, haemoglobin (115 vs 135 g/L, p = 0.0001) and serum iron (10 vs 17 μmol/L, p = 0.0001) levels were lower in patients on haemodialysis than in healthy subjects.

In end-stage renal disease malnutrition reduces hyperhomocysteinemia, consequently, any decrease in total homocysteine levels may be a marker of increasing malnutrition and inflammation. This is supported by our observations that CRP negatively ($r = -0.23$, $p = 0.02$), haemoglobin ($r = 0.33$, $p = 0.0001$) and albumin ($r = 0.32$, $p < 0.001$) levels positively correlated with total-homocysteine levels in haemodialysis patients. However, in healthy subjects without malnutrition and generalized inflammation, homocysteine levels correlated positively with IL-6 levels ($r = 0.33$, $p = 0.01$). This may suggest that increasing homocysteine levels, albeit in the normal range, have adverse effects on inflammation.

Serum cobalt, copper and nickel levels were significantly higher in haemodialysis patients than in healthy subjects: 0.3 vs 0.15 μg/L, p = 0.0001; 1214 vs 990 μg/L, p = 0.05; and 3.2 vs 2.1 μg/L, p = 0.04, respectively. Results both consonant with and contradicting to our observations can be found in the literature for all three elements. We

assume that cobalt, copper and nickel accumulations are strongly dependent on environmental factors.

In haemodialysis patients total homocysteine levels were negatively correlated with serum nickel ($r = -0.29$, $p < 0.01$), but not with serum cobalt and copper. Based on these findings we studied the possible effect of nickel on homocysteine metabolism in humans.

Homocysteine metabolism of quiescent peripheral blood mononuclear cells

Peripheral blood mononuclear cells (T lymphocytes, B lymphocytes, NK cells, monocytes) can be separated easily and fast from the whole blood. Methionine cycle is working in these cells like in any other mammal cells. It was demonstrated previously that proliferation of stimulated peripheral blood mononuclear cells leads to increasing homocysteine production, which may contribute to the development of hyperhomocysteinemia associated with immune activation. However, little was known about homocysteine metabolism, especially about the transsulfuration pathway in quiescent peripheral blood mononuclear cells. We found that the quiescent peripheral blood mononuclear cells *in vitro* continuously produce homocysteine, and release it to the medium reaching a balance between intra- and extracellular homocysteine levels. The radioactive methyl group from ^{14}C-methionine incorporated into the DNA and occurred in the lipids of the quiescent cells, confirming the activity of the transmethylases. It is known that methionine supplementation significantly increases the homocysteine production of stimulated peripheral blood mononuclear cells. We found that the homocysteine production of quiescent cells was also methionine dependent. Methionine

synthase activity in cell lysate significantly increased only due to the withdrawal of methionine (115±8%, $p < 0.05$), presumably in accordance with the increased need for methionine regeneration; this indicates the stability of remethylation potential in sufficient methionine status.

Cysteine withdrawal and addition of L-homocysteine (100 μmol/L) decreased (20±5% of control, $p < 0.001$, and 65±19% of control, $p < 0.04$, respectively), while addition of glutathione (100 μmol/L) increased (115±30% of control, $p < 0.05$) intracellular homocysteine concentrations. These findings suggest that the transsulfuration pathway is active in quiescent cells. We confirmed CBS mRNA expression in quiescent peripheral blood mononuclear cells of 10 healthy volunteers (CBS/GAPDH mRNA ratio: 0.964 ± 0.28). Then, we confirmed the CBS protein expression in stimulated and quiescent peripheral blood mononuclear cells using Western blot. The human hepatocarcinoma cell line HepG2 served as a positive control for CBS expression. CBS activity in quiescent peripheral blood mononuclear cells positively correlated with CBS protein levels normalized for GAPDH protein levels ($r = 0.526$, $p = 0.001$). CBS protein level and activity increased with incubation time and stimulation. SAM is the allosteric activator of CBS; adding it to the reaction we found marked elevation of CBS activity to 202±25% of control ($p < 0.001$).

We conclude that peripheral blood mononuclear cells may contribute to the plasma homocysteine level during stimulation as well as in quiescent state. In pathological conditions e.g. in renal diseases elevated oxidative stress and accumulated uremic toxins can significantly influence the homocysteine metabolism of circulating, quiescent peripheral blood mononuclear cells. By the expression of CBS, the key enzyme in homocysteine catabolism, quiescent peripheral mononuclear cells can

contribute to the elimination and detoxification of homocysteine in hyperhomocysteinemia.

The effect of trace elements on the homocysteine metabolism of peripheral blood mononuclear cells

We studied the effects of nickel at concentrations measured in sera of haemodialysis patients and healthy subjects on the homocysteine metabolism of peripheral blood mononuclear cells. In our studies water soluble nickel(II) was used at concentrations found in healthy subjects and haemodialysis patients (1-10 μg/L). We found concentration dependent nickel-induced decrease in intracellular homocysteine, glutathione and SAH levels up to 79±6, 70±13 and 85±10 % of controls, respectively, during 24-hour incubation, while intracellular concentrations of SAM and levels of thiols in the supernatant did not change significantly. Nickel was known to induce ROI formation and glutathione depletion, so the study was repeated in the presence of reduced glutathione concentration (100 μmol/L); this counteracted the effect of nickel. We found that nickel, up to 25μg/L concentration, did not modify cell viability or IL-2, TRAIL and FasL release of quiescent and stimulated peripheral blood mononuclear cells. Despite the nickel-induced elevation of SAM/SAH ratio, nickel-induced alteration in DNA methylation was not found. Nickel in the range of 1-25 μg/L, induced intracellular ROI formation in quiescent peripheral blood mononuclear cells in a concentration dependent manner. CBS enzyme is known to have a key role in maintaining the glutathione-dependent redox homeostasis; CBS activity is regulated by intracellular ROI through the heme cofactor. In nickel treated quiescent and stimulated peripheral blood mononuclear cells, we observed an increase in CBS

activity. Nickel treatment did not modify protein levels and did not induce ROI-mediated endoproteolyis of the CBS enzyme, confirmed by Western blot analysis. Experiments were also performed using cell lysate of freshly isolated peripheral blood mononuclear cells. Nickel added directly to the reaction mixture stimulated CBS activity (207±60% of control, $p < 0.01$), which was additive to the SAM induced stimulation. Thus, nickel enhances CBS activity, which remains sensitive to SAM binding. Theoretically, nickel-induced generation of low level ROI can stimulate CBS activity via oxidation of heme iron.

Summary

Hyperhomocysteinemia is an independent cardiovascular risk factor. We confirmed in the studied population that decreasing renal function impaired homocysteine metabolism, and haemodialysis and vitamin supplementation failed to normalize hyperhomocysteinemia. Our data point to the fact that amelioration of hyperhomocysteinemia in end-stage renal patients is often the result of increasing malnutrition and inflammation, which counteract the benefits of the reduction of homocysteine levels.

Haemodialysis patients are at risk for both deficiency of essential trace elements and excess of toxic trace elements. We found nickel, cobalt and copper accumulation in haemodialysis patients. We were the first to demonstrate that significant inverse correlation exists between serum nickel and plasma total homocysteine levels.

To study the effects of trace elements, quiescent peripheral blood mononuclear cells were used. We demonstrated that these cells expressed the active CBS enzyme essential for the first step of the homocysteine

catabolizing transsulfuration pathway. Thus, peripheral blood mononuclear cells in quiescent state can contribute significantly to the homocysteine homeostasis. Our cell system is a useful model for the study of homocysteine metabolism including interindividual differences.

We proved that homocysteine metabolism of quiescent peripheral mononuclear cells can be affected by nickel in the physiological concentration range. We found that the mechanism of nickel-induced CBS activity increase was mediated by increased ROI formation, most probably via the heme-binding domain of the enzyme. Increase of nickel concentration in haemodialysis patients may stimulate the elimination of homocysteine. However, putative mechanism of nickel action may contribute to the increase of oxidative stress, and may lead to unfavourable changes in iron profile.

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List of publications related to the dissertation

1. **Katkó, M.**, Zavaczky, E., Jeney, V., Paragh, G., Balla, J., Varga, Z.: Homocysteine metabolism in peripheral blood mononuclear cells: Evidence for cystathionine beta-synthase activity in resting state.
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List of other publications

3. Zsíros, N., Koncsos, P., Lőrincz, H., Seres, I., **Katkó, M.**, Szentpéteri, A., Varga, V.E., Fülöp, P., Paragh, G., Harangi, M.: Paraoxonase1 arylesterase activity is an independent predictor of myeloperoxidase levels in overweight patients with or without cardiovascular complications. *Clin. Biochem. "Accepted by Publisher" (2016)*
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