Influence of Cr(III)-picolinate, and Cr(III)-nicotinate on apoptosis induction in HBL-100 human mammary epithelial cells

Wpływ pikolinianu oraz nikotynianu chromu na apoptozę w ludzkich nabłonkowych komórkach gruczołu mlekowego linii HBL-100

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Wprowadzenie. Chrom trójwartościowy – Cr(III), jest niezbędnym dla organizmu mikroelementem, który uczestniczy w metabolizmie węglowodanów, tłuszczów oraz białek u ludzi i zwierząt. Wykazano, że niedobór Cr(III) występuje w cukrzycy typu II, chorobach układu krążenia oraz przy zaburzeniach układu nerwowego. W dostępnych na rynku suplementach diety Cr(III) występuje najczęściej w postaci pikolinianu, nikotynianu lub chlorku chromu; jednakże istnieją doniesienia pokazujące cytotoksyczne oddziaływanie Cr(III) w organizmie.

Cel. Ocena wpływu dwóch form Cr(III): pikolinianu chromu oraz nikotynianu chromu na indukcję apoptozy w ludzkich komórkach nabłonka gruczołu sutkowego linii HBL-100.

Materiały i metody. Ludzkie komórki nabłonka gruczołu sutkowego linii HBL-100 inkubowano w pożywce z dodatkiem pikolinianu lub nikotynianu chromu (obydwa podawane w stężeniu 10 µg/L) przez 1, 3 lub 6 godzin. Przy użyciu laserowej cystometrii skaningowej oznaczono liczbę komórek apoptotycznych na podstawie wielkości populacji komórek o zawartości DNA mniejszej niż w fazie G1 (pik sub-G1) oraz ekspresji wskaźników apoptotycznych: białka BAX i 89 kDa fragmentu polimerazy polyADP-rybozy (PARP), wybarwianych w komórkach metodą immunofluorescencji.

Wyniki. Wykazano, że pikolinian chromu indukował apoptozę w komórkach HBL-100, powodując zwiększoną ekspresję BAX i 89 kDa fragmentu PARP. Odnotowano również podwyższony procent komórek w fazie subG1 na histogramie DNA. Nikotynian chromu nie wywierał proapoptotycznego wpływu na komórki, powodując jedynie nieistotne statystycznie podwyższenie poziomu białka BAX.

Wnioski. Otrzymane wyniki wskazują na ryzyko indukcji apoptozy w ludzkich komórkach nabłonkowych eksponowanych na działanie nawet niewielkich stężeń (10 µg/L) pikolinianu chromu.

Słowa kluczowe: *pikolinian chromu, nikotynian chromu, apoptoza, BAX, PARP*

Introduction. Trivalent Chromium, Cr(III), is an essential trace element that participates in the carbohydrate, lipid and protein metabolism in humans and animals. The Cr(III) deficiency is shown to be associated with type 2 diabetes, cardiovascular diseases and nervous system disorders. Dietary Cr(III) supplements available on the market usually contain Cr in the form of chromium picolinate, chromium nicotinate, or chromium chloride; however, some reports indicate cytotoxic effect of Cr(III).

Aim. To evaluate the influence of two different Cr(III) compounds: Cr-picolinate and Cr-nicotinate on induction of apoptosis in human mammary epithelial cell line HBL-100.

Material & Method. HBL-100 human mammary epithelial cells (MECs) were treated with Cr-picolinate or Cr-nicotinate (both at concentration of 10 µg/L) for 1, 3 or 6 h. Laser scanning cytometry (LSC) was used to determine the number of apoptotic cells based on the size of the subG1 peak on DNA histogram, as well as the expression of apoptotic markers: BAX and 89 kDa fragment of cleaved PARP {poly(ADP-ribose)polymerase} determined by immunofluorescence staining.

Results. The results showed that Cr-picolinate stimulated the induction of apoptosis in human MECs, which was manifested by significantly higher percentage of cells in the subG1 phase of cell cycle, elevated expression of BAX protein, as well as augmented level of the cleaved 89 kDa PARP fragment. Cr-nicotinate did not reveal such effect, causing only slight elevation of BAX level.

Conclusion. The results of the present study point at the risk of apoptosis induction in human epithelial cells exposed to Cr-picolinate even at concentrations as low as 10 µg/L

Key words: *chromium picolinate, chromium nicotinate, apoptosis, BAX, PARP*

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Introduction

Trivalent and hexavalent chromium {Cr(III) and Cr(VI), respectively} are the most common forms of chromium in the environment; however, their effect on living organisms is quite inverse. Cr(VI) has been classified by International Agency for Research on

Cancer (IARC) as a Class I Carcinogen [1]; whereas Cr(III) is considered to be relatively less toxic. Cr(III) is also an essential trace element as it participates in carbohydrate, lipid and protein metabolism in humans and animals. The Cr(III) deficiency was shown to be associated with type 2 diabetes, cardiovascular diseases and nervous system disorders. Supplementation of patients suffering from type 2 diabetes with chromium (III) supplements bring about beneficial effects on glucose, glycosylated hemoglobin, insulin and cholesterol levels [2]. Nowadays, dietary Cr(III) supplements are commonly available on the market. These supplements usually contain chromium in the form of chromium picolinate {$Cr(III)(pic)_3$, Cr-picolinate} and chromium chloride {$Cr(III)Cl_3$}. Cr-picolinate is often used in treatment of type 2 diabetes due to its solubility and increased absorption efficiency [3]. On the basis of data obtained from human clinical trials, the Council of Responsible Nutrition, Washington, USA, has stated that the safety level of Cr(III) was up to 1000 μg/day which included the picolinate forms and other forms of Cr(III) [4]. However, the American Food and Drug Administration agency (FDA) has not approved Cr-picolinate as a food additive nor has it listed $Cr(III)(pic)_3$ under the "Generally recognized as Safe" (GRAS) category [5]. The reason for that may be ambiguous *in vivo* and *in vitro* results of studies on the effect of this form of Cr(III) in living organisms. Some of them report safe tolerance while others describe elicitation of toxicity. Hepburn and Vincent [6] demonstrated increased lipid peroxidation in liver and kidney in rats supplemented with Cr-picolinate, indicating oxidative damage caused by this compound. Similar oxidative damage was noted in cultured macrophage J774A treated with Cr-picolinate [7]. Furthermore, some studies have shown an interaction of Cr-picolinate with DNA. A mutagenic effect of Cr-picolinate used in the range of 0.2-1.0 mM was reported, causing DNA fragmentation in macrophage J774A1 cells [8]. Bonding Cr^{+3} with picolinic acid makes chromium much more genotoxic than the other forms of this metal. Hepburn et al. [9] observed that Cr-picolinate increased lethal mutations and dominant female sterility in a multi-generation *Drosophila* study. A study on peripheral blood lymphocytes also demonstrated cytotoxic activity of Cr(III) $(pic)_3$, resulting in induction of apoptotic cell death through formation of reactive oxygen species (ROS) and activation of mitochondrial pathway [10].

Thus, the aim of the present study was to evaluate the influence of two different Cr(III) compounds: Cr-picolinate and Cr-nicotinate on induction of programmed cell death in human mammary epithelial cell line HBL-100. The number of apoptotic cells, as well as expression of proapoptotic protein BAX, and the level of 89 kDa fragment of PARP (poly(ADP-ribose) polymerase), which is regarded as a marker of apoptosis, have been evaluated in HBL-100 cells treated with Cr-picolinate, Cr-nicotinate, or their respective acids (picolinic acid, nicotinic acid).

Aim

To evaluate the influence of two different Cr(III) compounds: Cr-picolinate and Cr-nicotinate on induction of apoptosis in human mammary epithelial cell line HBL-100.

Material and method

Media and reagents

DMEM medium with L-glutamine and sodium pyruvate, phosphate buffer saline, pH=7.4 (PBS), fetal bovine serum (FBS), fungizone, gentamycin sulphate were obtained from Life Technologies (Carlsbad, CA, USA), rabbit anti-PARP was supplied by Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Monoclonal mouse anti-BAX was purchased from Oncogene Research Products (San Diego, CA, USA). Alexa Fluor 488 chicken anti-mouse secondary antibody and Alexa Fluor 488 chicken anti-rabbit secondary antibody were purchased from Life Technologies, Molecular Probes (Eugene, OR, USA). Sterile conical flasks, 8-chamber culture slides and sterile disposable pipettes were purchased from Nunc Inc. (Naperville, IL, USA).

Cell culture

Human mammary epithelial cell line (HBL-100) was obtained from the American Type Culture Collection (Rockville, MD, USA). Cell cultures were maintained in DMEM supplemented with 10% (v/v) FBS, 50 μg/ml gentamycin, 2.5 μg/ml fungizone, 50 UI/ml penicillin and 50 μg/ml streptomycin in an atmosphere of 5% CO_2/95% humidified air at 37°C, and routinely subcultured every 2 or 3 days.

Chromium

Picolinic and nicotinic acids as well as chromium salts used in this experiment were obtained from Sigma-Aldrich (St. Louis, MO, USA). All used chrome forms were diluted to the final concentration of 10 μg/L of Cr(III). Nicotinic acid and picolinic acid were diluted in the culture medium. Cr-picolinate and Cr-nicotinate were dissolved in DMSO.

Experimental procedure and immunofluorescence staining

Exponentially growing HBL-100 cells were harvested using trypsinization method, and plated on the

8-chamber culture slides at a concentration of 25000 cell/well of chamber slide. The cells were cultured for 24 h and then incubated with the investigated substances in 10% FBS/DMEM for 1, 3 and 6 h. The control cells were cultured in 10% FCS/DMEM supplemented with equivalent concentrations of DMSO. Then cells were fixed in 0.25% formaldehyde for 15 min, washed twice with PBS, suspended in ice-cold 70% methanol and incubated at 4°C for 30 min. Afterwards methanol was aspirated and samples were washed twice with PBS containing 1% BSA (bovine serum albumin) (w/v) and incubated for 1 h with chosen primary antibody diluted 1:250 in PBS-1% BSA. After the primary incubation the cells were washed twice with PBS-1%BSA, and incubated for 1h with 1:500 secondary antibodies. The cells were then washed twice in PBS-1%BSA and finally incubated with 5 µg/ml solution of 7-aminoactinomycin D (7-AAD) for 30 min to counterstain the DNA. Then the chamber walls were removed and coverslips were mounted on microscope slides using an anti-fade mounting medium (ICN Biomedicals Inc., Aurora, OH, USA).

Laser scanning cytometry

The probes were analyzed by a laser scanning cytometer (LSC) (CompuCyte Corp., Boston, MA, USA). At least 5 x 10^3 cells per chamber area were analyzed. The fluorescence excitation was provided by a 488 nm, 10 mW Argon laser beam. The green fluorescence of Alexa Fluor 488-labeled antibody was measured using a combination of dichroic mirrors and filters transmitting at 520±20 nm wavelength (detector offset and gain set to 2000 and 32, respectively), and far red fluorescence of 7-AAD transmitting at >650nm (offset 2000 and gain 30). Percent of apoptotic cells (subG1) was measured as a percent of cells with lower concentration of DNA and highest value of red fluorescence in the cell. Another parameter measured was high maximal pixel of BAX and PARP fluorescence (HMP) corresponding to the highest value of fluorescence in the cell, regardless of cellular compartment.

Statistical evaluation

The results were statistically evaluated by ANOVA and Tukey's multiple range tests using Prism version 2.00 software (GraphPad Software, San Diego, CA). P value of $\leqslant 0.05$ was regarded as significant; n=12.

Results

LSC scans of HB-100 cells treated with different compounds containing Cr(III) revealed an increase in apoptotic cell number (measured by the percentage of cells in subG1 phase of cell cycle) after exposure to Cr-picolinate. The number of apoptotic cells after the first hour of incubation with Cr-picolinate reached 5% of all analyzed cells, while at the 6th h of treatment it amounted to 32% (Fig. 1a).

The second tested form of Cr(III) – Cr-nicotinate evoked similar results after the first hour of treatment, but a significantly lower percentage of cells in subG1 phase after 6 h (12%) (Fig. 1a). The increase of the

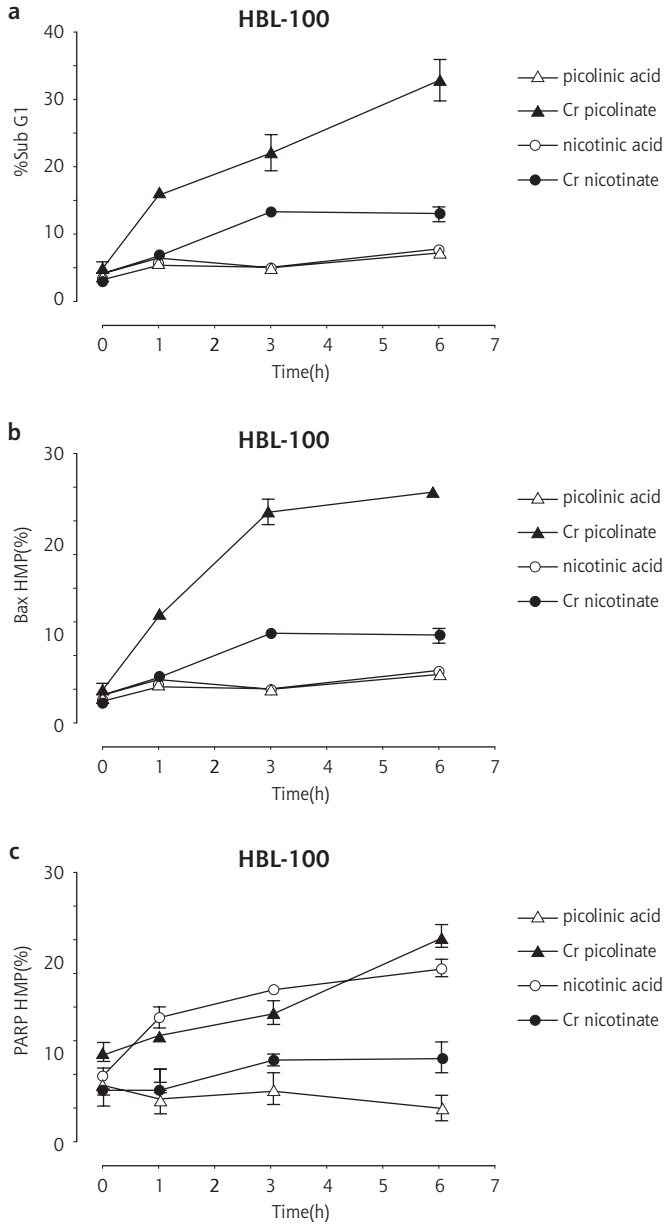

Fig. 1. Effect of 1, 3, and 6 h treatment of HBL-100 human mammary epithelial cells with two forms of Cr(III): Cr-picolinate and Cr-nicotinate on apoptosis induction; a. percent of apoptotic cells ± SEM recognized in the subG1 phase of cell cycle of HBL-100 cells treated with Cr-picolinate and Cr-nicotinate (10 µg/L of Cr^{3+} concentration), and respective acids (picolinic and nicotinate acid); b. changes in the percentage of cells showing high expression of proapoptotic protein BAX after treatment of HBL-100 cells with Cr-picolinate, Cr-nicotinate, picolinic acid, and nicotinic acid; c. changes in the percentage of cells showing high expression of PARP after treatment of HBL-100 cells with Cr-picolinate, Cr-nicotinate, picolinic acid, and nicotinic acid.

percentage of apoptotic cells in the tested cell line after picolinic acid and nicotinic acid was statistically insignificant. Furthermore, after the 6 h incubation with Cr-picolinate a significant increase in BAX HMP (high maximal pixel) from 10 to 25% was observed in HBL-100 cells (Fig. 1b). Changes in PARP HMP occurred rapidly after 1h of incubation and further increased with the time of treatment (from 7% at 1st h to 66% after 6 h) (Fig. 1c). This rapid increase of BAX and PARP HMP within 1h of incubation of MECs with Cr-picolinate was parallel to the increase in the number of cells in the subG1 region of cell cycle which represented apoptotic cells (Fig. 1a). Interestingly, the treatment of HBL-100 cells with picolinic acid also increased the levels of BAX; however, the values of BAX HMP reached only half of the values observed for Cr-picolinate (Fig. 1b). On the other hand nicotinic acid alone caused increased levels of 89 kD fragmnent of PARP, similarly to that induced by Cr-picolinate, and higher than the values obtained after the incubation of MECs with Cr-nicotinate (Fig. 1c).

Discussion

Chromium picolinate is a stable complex of trivalent Cr and it is the most popular form of chromium nutrient supplement. The present *in vitro* study showed that treatment of human MECs with Cr-picolinate significantly increased participation of cells in the subG1 phase of cell cycle, indicating induction of apoptosis. During programmed cell death the DNA degradation to fragments 50-300 of kilo bases occurs. These fragments undergo internucleosomal cleavage and multiplication. The rise of such DNA aggregation is detectable by laser scanning cytometry (LSC) as subG1 area of DNA histogram and an increase in the number of cells having DNA maximal pixels of fluorescence originating from a counterstain with 7-AAD. Such results indicate that this chromium compound shows apoptogenic properties. These observations are in agreement with the results presented by Petit, et al. [11] who showed implication of Cr(III) ions in induction of apoptosis in macrophages. The human macrophage U937 cell line treated with Cr(III) ions inhibited Bcl-2 expression and stimulated BAX as well as caspase-3 and 8 expressions. Another study by Shrivastava et al. [12] on dermal skin fibroblasts demonstrated that exposition of these cells to different Cr(III) complexes caused morphological changes (cellular blebbing, formation of apoptotic bodies, chromatin condensation) reflecting substantial cellular damage.

Balamurugan at al. [13] have shown a crucial role of caspase-3 in the Cr(III) induced lymphocyte apoptosis. This effect was connected with mitochondrial damage. Picolinate ligands shift the redox potential of chromic center in such a way that it is susceptible to reduction of another atoms or particles producing reactive oxygen species (ROS). The fact that only Cr-picolinate but not Cr-nicotinate or chloride possesses such properties suggests importance of ligand in apoptosis induction by chromium (III). This thesis is in agreement with our results, showing that Cr-nicotinate caused only an insignificant increase of the number of HBL-100 cells in the subG1 phase. Interestingly, in the latest study on chick embryo fibroblasts, the cytotoxic effect of Cr-picolinate was evoked only when high concentrations of this compound (400-600 μM) were applied, causing an increase in ROS production, alternations of mitochondrial membrane potential, and apoptosis. On the other hand, low concentrations of Cr-picolinate (8-16 μM) did not cause such changes [14]. In the present study Cr-picolinate was administered to human MECs at very low concentration of 80 μg/L (equivalent of 0.19 μM), which was sufficient to induce apoptosis.

The mechanism by which Cr-picolinate induces apoptosis is not fully understood. Rudolf and Cervinka [15] used a model of human primary skin fibroblasts to study pathways activated by trivalent chromium, and proposed a possible sequence of events. Their research revealed several targets of Cr(III) actions, including Rac-1 and Src kinases, which in turn activate p38 and JNK kinases, resulting in activation of caspase-3, a well-recognized executory enzyme of apoptosis. The authors also noted elevated levels of oxidative stress and more massive damage resulting in necrosis.

BAX (Bcl-2-associated X protein) is known as a strong inducer of apoptosis controlled by the transcription factor p53. The estimation of BAX expression in cells treated with Cr-picolinate revealed elevated aggregation of this protein from 10 to 25% in HBL-100 cells. BAX protein plays the main role in mitochondrial apoptotic pathway, together with Bid and VDAC, which participate in the building of omega channels in external mitochondrial membrane. Not only the BAX aggregation was observed in the used cell line exposed to Cr-picolinate treatment. Additionally, the cleavage of poly(ADP-ribose)polymerase (PARP) was noted. In spite of the fact that PARP is involved in reparation of damaged DNA and is activated by DNA strand breaks, its proteolytic cleavage into 89 kDa fragment is closely associated with the apoptosis induction. This cleavage is thought to be a regulatory event for cellular death. Our results indicated an increased aggregation of this protein in cells treated with Cr-picolinate. This suggests that during the incubation with Cr-picolinate the cellular processes leading to cell death were activated. Also the *in vitro* study performed on J774 macrophages revealed induction of PARP

cleavage after 6 h of incubation with Cr(III) ions used in high concentration (up to 500 mg/L) [16].

In the study by Berner et al. [17] evaluating chromium tripicolinate safety, the authors concluded that this compound was considered safe for intended use up to the maximal level of 2.4 mg Cr picolinate per serving. The lack of Cr(III) toxicity (administered in the form of chloride or picolinate) was also shown in a study by Anderson et al. [18] who fed rats with a diet enriched with Cr(III) up to 100 mg/kg for 20 weeks, and observed no changes in biochemical and histological parameters of blood. Such a high chromium intake calculated per kg BW is over a thousand times higher than the upper limit of the estimated safe and adequate dietary intake for humans (200 µg/d; NRC 1998). The WHO [19] estimated that 33 µg Cr/d was an adequate dietary daily intake of Cr. It was shown that very often to reduce insulin resistance and the effects of type 2 diabetes, and to attenuate the risk of cardiovascular diseases, much higher doses of Cr^{+3} were used (up to 1 mg/d). However, such a long-lasting consumption of trivalent chromium supplements in doses several times higher than the recommendations may cause a negative impact on human organism. It was shown that in patients who consumed up to 2.4 mg of Cr^{+3} each day for several months some liver and kidney disorders appeared [20]. Hepburn and Vincent [21] found that supplemental $^{51}Cr(pic)_3$ was entering tissue cells intact and in hepatocytes radiolabel was first noticed in nucleus and mitochondria than in cytosol. However, short life-time of this compound in the body (less than 1 d) reduced the potential DNA damage.

Conclusion

The results of our study indicate that even a low concentration of Cr(III) (10 µg/L) in the form of Cr-picolinate [80 µg/L of $Cr(picol)_3$] may induce apoptosis in epithelial cells. Cr-nicotinate did not show significant effect on the apoptosis stimulation. The obtained results point at the risk of the apoptosis induction in human epithelial cells exposed to Cr-picolinate.

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