ZINC CONTENT IN THE DIET AFFECTS THE EXPRESSIONAL CHANGES OF CU/ZNSOD IN AORTA OF SPONTANEOUSLY HYPERTENSIVE RATS

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Reviewed by: Assoc. Prof. N. Negrev, MD, PhD

SUMMARY

Oxidative stress induced by reactive oxygen species (ROS) plays an important role in development of hypertension. Vascular cells have a complex antioxidant system for protection against increased oxidative stress. Zinc is a cofactor of one of the most important antioxidant enzyme – copper-zinc superoxide dismutase (Cu/ZnSOD). The aim of the present study was to examine the effects of feeding different Zn containing diets (40, 60, 160 mg Zn/kg lab chow) on the activity and expression of Cu/ZnSOD in aorta of male (n=27) spontaneously hypertensive rats (SHR). The diets were introduced at the beginning of the development of hypertension (2 months after birth) and the animals were fed for 8 weeks. Cu/ZnSOD expression was analyzed by immunohistochemistry and the activity was measured by RANSOD kit (RANDOX). Atomic-absorption spectrometry was used to determine Zn and Cu concentrations in the rat's sera. Cu/ZnSOD was mainly expressed in medial smooth muscle cells and it had a weak immunoreactivity in the endothelium. In the group with Zn supplementation diet (160 mg Zn/kg lab chow), Cu/ZnSOD staining was more enhanced in the smooth muscle cells and endothelium, and the systolic blood pressure was significantly decreased ($P<0.05$) in comparison with the groups fed with other Zn diets (40 or 60 mg/kg lab chow). The change in Cu/ZnSOD activity correlates well with the protein expression and with the arterial blood pressure alterations. The present data suggest that Zn concentration in the diet may play an important role in the regulation of the blood pressure and may in part be a critical nutrient for maintenance of anti-oxidative events in the endothelial cells in SHR.

INTRODUCTION

Zinc is a critical component of biomembranes and is essential for proper membrane structure and function and the activity of numerous enzymes (Oteiza PL, 1996). It could conceivably limit oxidant-induced damage. Some possible antioxidant actions of zinc include: a) Stabilization of membrane structure (Di Silvestro R., 2000); b) Restriction of endogenous free radical production (Sakanashi TM, 1993); c) Contribution to the structure of the antioxidant enzyme extracellular superoxide dismutase (Miller AF., 2004); d) Maintenance of tissue concentrations of

metallothionein, a possible scavenger of free radicals (Cousins RJ., 1995).

It has been shown that Zn deficiency aggravates arterial blood pressure in SHR rats by decreasing Cu/ZnSOD activity and increases lipid peroxidase, which is a binding segment between hypercholesterolemia and atherosclerosis (Khoja SM, 2002; Mertens A, 2001). There are also data indicating that changes in the serum zinc concentrations can alter CuZnSOD activity, cholesterol (Chol), and triglycerides (Tg) concentrations, and the level of mmLDL in rats (Yousef MI, 2002).

The present study focused on the effect of Zn containing diets on the activity and expression of Cu/ZnSOD, arterial blood pressure, lipid peroxides (ROOH), lipid variables (LDL, HDL, triglycerides and cholesterol) in male SHR rats.

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MATERIALS AND METHODS

The investigation conforms to the Guide for Animal Care and Use of Laboratory Animals by the Ethical Committee of the Medical University, Pleven, Bulgaria

Animals and diets

Male spontaneously hypertensive rats (SHR, n=27), with b.wt. 212.84 ± 22.54 were used. Animals were housed in groups of 5 per cage and kept under a normal 12 h light/dark cycle at $22^\circ \pm 2^\circ\text{C}$. Rats were allowed access to food and water *ad libitum*. They were randomly divided into 3 groups: Group 1 (G1, n = 10) - 40 mg Zn /kg diet; Group 2 (G2, n = 7) – 60 mg Zn /kg diet; Group 3 (G3, n = 10) - 160 mg Zn/kg. The zinc diets containing different Zn amounts were introduced at the beginning of the development of hypertension (2 months after birth) and the animals were fed for 8 weeks.

Zinc content in the laboratory chow and serum concentration of zinc and copper were analyzed by a flame atomic-absorption spectrophotometry, using Perkin-Elmer, Model-Analyst 300 apparatus.

Blood Collection and abdominal artery preparation.

At the end of the treatment period, following overnight fasting, the abdominal cavity of rats was opened under pentobarbitone sodium anesthesia (26 mg/kg body weight, i.p.). Blood was collected from the bifurcation of the aorta for the measurements of Zn and Cu, SOD activity, lipid profile. The abdominal artery was post-fixed by immersion in 10% neutral buffered formalin (NBF) and embedded in paraffin.

Immunohistochemistry

Consecutive sections (5 μm thick) of paraffin-embedded abdominal arteries were cut, and immunostained with goat anti-rabbit CuZnSOD (1:500 dilution, Santa Cruz Biotechnology, USA), antibody by using an avidin–biotin–peroxidase complex (ABC) method (DAKO, USA) for 30 minutes, according to the manufacturers' instructions.

Table 1. Levels of Zn and Cu, serum ROOH and Cu/ZnSOD activity in SHR fed a standard (G1), G2 and a Zn-supplement diet (G3) for 8 weeks.

Zn concentration in the diet [mg/kg]	Zn [mmol/L]	Cu [mmol/L]	ROOH [nmol/ml serum]	SOD activity [U/g Hg]
G1 - 40	18.92 ± 0.43	27.33 ± 1.18	4.61 ± 0.31	2049.1 ± 257.7
G2 - 60	19.86 ± 0.51	26.16 ± 1.29	4.86 ± 0.29	2177.1 ± 337
G3 - 160	$22.24 \pm 0.28^*, \#$	25.96 ± 0.33	$3.48 \pm 0.08^*, \#$	$3179.3 \pm 286.1\#$

Data reported represent means $\pm$ S.E. of the values. * - $p < 0.05$; # - $p < 0.001$

* - significant difference between G2 and G3; # - significant difference between G1 and G3

All sections were counterstained with haematoxylin and examined by light microscopy OLYMPUS BX40.

Chemical analysis

Assay of Cu/ZnSOD activity

Following blood collection and centrifugation at 3000 rpm for 10 minutes, the plasma was aspirated and the erythrocytes were rinsed four times with 3 ml saline solution at 4°C . Measurement of erythrocytes CuZnSOD activity was determined by RANSOD reactive photometric method (RANDOX, England).

Cu-Zn Superoxide dismutase measurement

This method employs xanthine and xanthine oxidase (XOD) to generate superoxide radicals, which react with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) to form a red formazan dye. The superoxide dismutase (SOD) activity is then measured (at 505 nm) by the degree of inhibition of this reaction. One unit of SOD is that which causes a 50% inhibition of the rate of reduction of INT under the conditions of the assay.

Lipid analysis

Lipid analysis was performed on a Hitachi-704 automated clinical chemistry analyzer (Boehringer, Germany) with reagents from Horiba ABX Diagnostics (France). Total cholesterol was detected by enzymatic photometric test (CHOD-PAP, Horiba ABX Diagnostics, France). Triglycerides and HDL-cholesterol determinations were done following the manufacturer's instructions (Horiba ABX Diagnostics, France). The concentration of LDL was determined following the method described by Friedewald W.T. (1972).

$$LDL = \text{total cholesterol} - HDL - \frac{\text{triglycerides}}{2.2}$$

Blood pressure determinations

Blood pressure was determined by tail cuff plethysmography (Blood Pressure Recorder, Ugo Basile, Italy). Conscious rats were placed on a heated pad in a temperature-controlled quiet room. After a 15-min rest with the tail placed inside a tail cuff, the cuff was inflated three to four times to condition the animal to the procedure.

Degree of oxidative damage

The degree of oxidative damage was estimated by means of lipid peroxides (ROOH) in serum following the procedures described by Yagi (1987).

Statistical Analysis

Results from biochemical assays were expressed as the mean $\pm$ SE. The data were analyzed using one-way and two-way analysis of variance (ANOVA) when they were normally distributed, or with Kruskal-Wallis and medial test when they deviated from normal distribution. Descriptive statistics were used for immunohistochemical data, analyzing median, minimum, maximum and percentage. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Zn treatment on the level of Zn and Cu, serum ROOH and Cu/ZnSOD activity

Table 1 shows data on the level of Zn and Cu, serum ROOH and SOD activity in SHR fed a standard (G1) or a Zn-supplement diets (G2 and G3) for 8 weeks. The Zn-supplement diet group (G3) had a significantly increased concentration of serum Zn relative to the standard diet group (G1). There was no significant difference in serum Cu concentration between the groups.

Cu/ZnSOD activity

The effect of Zn diets on CuZnSOD activity in the red blood cells was investigated to determine whether superoxide radicals in/and around arterial endothelia cells affect vascular resistance and blood pressure. Our result showed that the activity of Cu/ZnSOD was higher in G3 and decreased in G1. There was a significant difference in the activity of G3 compared to G1 ($p = 0.020$). The data for activation of CuZnSOD correlated positively with the application of increasing Zn concentration in the diets ($r = 0.501$, $p = 0.008$).

ROOH

We monitored the level of serum ROOH and determined that it depended on the zinc diets. SHR rats treated with the lower Zn diet (G1) showed significantly increased concentration of serum lipid peroxides in comparison with animals treated with higher Zn concentration (G3, $p = 0.016$). We have also observed significant difference in the concentration of lipid peroxides between G2 and G3 ($p = 0.005$). The level of serum ROOH correlated negatively with the Zn concentration in the diets ($r = -0.512$, $p = 0.015$).

Effect of Zn treatment on rat systolic blood pressure

Table 2 summarizes the effects of Zn administration on systolic blood pressure (SBP) of SHR. All groups of SHR showed continues increase of systolic blood pressure fol-

lowing the administration of different Zn containing diets, reaching significance on month 4 ($p < 0.001$, compare to SBP on month 2).

Table 2. Systolic blood pressure obtained from SHR fed a standard (G1), diet 2 - 60 mg Zn/kg and a Zn-supplement diet (160 mg Zn/kg).

Zn concentration in the diet [mg/kg]	Blood pressure (mm Hg) - Month 2	Blood pressure (mm Hg) - Month 4
G1 - 40	175.1 $\pm$ 2.21	190.8 $\pm$ 2.37*, #
G2 - 60	169.85 $\pm$ 3.7	187.14 $\pm$ 3.75*
G3 - 160	168.4 $\pm$ 3.32	178.8 $\pm$ 1.79*

Systolic blood pressure was measured as described in the Methods section.

* $p < 0.001$, # $p < 0.05$; * - significant difference between month 4 and month 2; # - significant difference between G1 and G3

Two months dieting with Zn at concentrations 160 caused blood pressure to be lowered significantly ($p < 0.01$) compare to control group (40 mg/kg diet). These observations show that for Zn at concentrations 160 mg/kg, the dosage and the duration of the regimen were effective in the obtaining a blood pressure decrease without severely compromising the health of the treated animals.

Lipid profile

Some investigators reported effects of zinc on lipid metabolism but the mechanism of action is not clear enough. The changes in serum total lipid values at 8 weeks after beginning of diet are given in Fig. 1. A significant decrease was observed in serum level of LDL ($p < 0.05$) in SHR fed a Zn diet 3 (G3), (0.49 ± 0.05) compared to SHR fed a Zn diet 1 (G1), (0.68 ± 0.04). No difference was found for triglyceride and total cholesterol between G1, G2 and G3. HDL was significantly increased in group 3 (G3), (0.64 ± 0.06) in comparison with group 2 (G2), (0.54 ± 0.03 ; $p < 0.05$) and group 1 (G1), (0.55 ± 0.02 ; $p < 0.05$).

Fig. 1. Serum lipid profile in SHR fed a standard (40 mg/kg), diet 2 - 60 mg Zn/kg and a Zn-supplement diet (160 mg Zn/kg).

Immunohistochemical protein localization of Cu/ZnSOD

Fig. 2. Microphotograph of immunohistochemical Cu/ZnSOD staining. (A) - Cu/ZnSOD negative control. Cu/ZnSOD was present in control group (diet with 40 mg Zn/kg lab chow - G1) (B) and in G2-60 mg Zn/kg lab chow (C) and G3 - 160 mg Zn/kg lab chow (D). Magnification x 400.

To realize how different Zn diets effect the distribution of CuZnSOD in the endothelium, an immunohistochemical studies were performed with the abdominal aorta (Fig. 2.), following 2 months of treatment. Immunohistochemical examination of the CuZnSOD showed that CuZnSOD was expressed mainly in medial smooth muscle cells.

was evaluated by immunohistochemistry. By immunohistochemistry, semiquantitative evaluation revealed that Cu,ZnSOD was present in control group and groups with Zn supplementation, but its expression increased according to the zinc diets, reaching the highest level in G3 (Table 3).

Table 3. Quantification of Cu/ZnSOD expression in control group G1 and groups G2 and G3.

Group	mean $\pm$ SE	Median (range)	Intensity1/ Percent (%)2	
G1-40 mg Zn/kg	1.30 $\pm$ 0.15	1.00 (1.00-2.00)	1	70
			2	30
G2-60 mg Zn/kg	2.00 $\pm$ 0.27	2.00 (1.00-3.00)	1	36.4
			2	27.3
			3	36.4
G3-160 mg Zn/kg	2.5 $\pm$ 0.15a	2.50 (2.00-3.00)	2	50
			3	50

^a $P < 0.05$ vs control. ($n = 7-10$ cases per group). ¹The intensity of immunohistochemistry was graded blinded according to the following scale: 0: absent, 1: weak, 2: moderate and 3: intense. ²Percentage of samples according to degree of staining.

CuZnSOD had only weak immunoreactivity in the endothelium following all treatments. CuZnSOD staining was increased with increasing of the Zn concentration in the diet.

To investigate the mechanisms leading to an increase of SOD activity in the red cells Cu,ZnSOD protein expression

DISCUSSION

The function of zinc in the body metabolism is based on its enzymatic affinity, such as a zinc-enzyme complex or Zinc metalloenzyme. Zinc is a required cofactor for a variety of antioxidant enzymes, particularly superoxide dismutase

There are varying reports on changes in plasma lipid peroxidation, zinc and erythrocyte Cu-Zn superoxide dismutase enzyme activity in SHR. Some of the studies reveal an increase, whereas others showed a decrease or no significant differences (Marjani A., 2005). There are a few reports describing differences in serum lipid peroxidation, zinc and erythrocyte Cu-Zn superoxide dismutase activity between SHR versus controls (WKY).

The results of this study show that in SHR fed a Zn diet 3 (G3) serum lipid peroxidation was significantly decreased, and that zinc levels were increased compared to controls ($P < 0.05$). This study also shows that the erythrocyte superoxide dismutase enzyme activity was increased in SHR fed a Zn diet 3 compared with controls ($p < 0.05$).

Cu/ZnSOD was mainly expressed in medial smooth muscle cells and it had a weak immunoreactivity in the endothelium. In the group with Zn supplementation diet (160 mg Zn/kg lab chow), Cu/ZnSOD staining was more pronounced in the smooth muscle cells and endothelium, and the systolic blood pressure was significantly decreased ($p < 0.05$) in comparison with the groups fed with other Zn diets (40 or 60 mg/kg lab chow).

A functional relationship is found between zinc content in diet, activity of Cu/ZnSOD, arterial blood pressure and lipid profile. Diet with high zinc content (160 mg/kg) causes increase in the activity of Cu/ZnSOD, decreases the blood pressure and changes in lipid variables. Zinc supplementation (160 mg/kg) affects lipid profile by decreasing LDL and increasing HDL.

By immunohistochemistry, semiquantitative evaluation revealed that Cu/ZnSOD was present in control group G1-40 mg Zn/kg lab chow, in G2-60 mg Zn/kg lab chow and G3-160 mg Zn/kg lab chow, but its expression increased according to the Zn diets, reaching the highest level in G3 (diet with zinc supplementation).

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