Regulation of zinc transporter 3 and carbonic anhydrases 2 and 14 mRNA expression in the retina of rats affected by low dietary zinc

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ABSTRACT. We looked at how zinc transporter 3 ZnT-3) mRNA expression in the rat retina is affected by low dietary zinc. Groups of 12 four-week-old male Sprague Dawley rats were fed on a low-zinc diet for 2, 4 or 6 weeks. Half of each group was then fed with a normal-zinc content diet and the other half was given a low-zinc content diet. The expression of ZnT-3, carbonic anhydrase 2 (CA2) and 14 (CA14) were detected by RT-PCR. After the rats were fed a low-zinc content diet for 2 weeks, their retina CA2 and CA14 mRNA levels were decreased, and the ZnT-3 mRNA was increased compared with the control rats. After they were fed a low-zinc diet for 4 weeks, ZnT-3, CA2 and CA14 mRNA levels decreased significantly. Then, after being changed back to a normal diet for 2 weeks, the rats had ZnT-3, CA2 and CA14 mRNA levels recovery in the retina.

Key words: Zinc deficiency; Zinc transporter 3; Carbonic anhydrase

INTRODUCTION

Normal ocular tissues contain relatively high levels of zinc, with a high percentage of this localized in the photoreceptors and retinal pigment epithelium (RPE) cells (Kokkinou et al., 2005). Recent studies show that intracellular localization of Zn^{2+} pools in photoreceptors changes with light exposure, with the greatest intensity of zinc staining observed in the perikarya of photoreceptors of dark-adapted retinas and in the inner segments of light-adapted retinas. Zn^{2+} movement between RPE and photoreceptors is also light-dependent, suggesting that Zn^{2+} is critical to normal visual function (Ugarte and Osborne, 2001).

Zinc provides structural stability to the Zn finger domains of many DNA-binding proteins and is a cofactor for more than 300 metalloenzymes, in which it is an essential element for the catalytic site of the enzymes or serves in a structural capacity to facilitate enzymatic function (McCall et al., 2000). Carbonic anhydrase (CA) comprises a family of zinc metalloenzymes, and there are 10 kinds of carbonic anhydrase isozymes (CAI, II, III, IV, VA, VB, VI, IX, XII and XIV) and 3 kinds of carbonic anhydrase protein (CA-PR VIII, X, XI).

CA2 and CA14 have been found to be the two zinc enzymes most closely linked to visual organization (Parkkila et al., 2001). Nagelhus et al. (2005) showed that CA XIV occurs in glial cells of the retina rather than in neurons and that it is highly expressed in the RPE, indicating that CA XIV is also involved in photoreceptor function in the retina. CA IV and CA XIV are membrane-bound carbonic anhydrases the play an important role in regulating the liquid around RPE cells. Ogilvie et al. (2007) found that the role of CA XIV of retinal function was more important than that of CA IV, and Lukaski (2005) showed that the expression levels of carbonic anhydrase changed with the amount of zinc in the body.

Zinc deficiency can produce adverse effects on visual function (Hyun et al., 2001; del Valle et al., 2003). Zinc deficiency is related to night blindness, tunnel vision, decreased visual acuity, and loss of color discrimination ability (Redenti and Chappell, 2002). Vinton et al. (1990) conducted vision research on 8 children who had no prior malnutrition, in which the majority of them were just left gifted with parenteral nutrition. The results showed that the eight children had ERG light response delay, where the majority of them had significant visual dysfunction. No such changes were found in concomitantly analyzed adults. With regard to six kinds of nutrients (vitamins A and E, zinc, selenium, linolenic acid, and taurine), plasma zinc level was the most relevant to visual function abnormalities, indicating that zinc deficiency affects visual development and retinal visual function to varying degree (del Valle et al., 2003).

Disruption of zinc homeostasis is strongly implicated in the pathophysiology of many chronic neurodegenerative diseases and acute neural injuries. Excessive and inadequate levels of bioavailable zinc are detrimental to the health of neurons. This release may account for the unusually high Zn content in the somata of neurons degenerating after severe episodes of ischemia or seizure activity (Capasso et al., 2005).

The major influx and efflux routes of Zn^{2+} are through two classes of multipass transmembrane proteins, ZnT and ZIP. At least nine ZnT and 14 ZIP transporters have been identified in human cells. These transporters exhibit tissue-specific expression and have unique responses to dietary zinc, hormones, and cytokines. They are located on plasma and vesicular membranes, and the two families have opposing functions in mediating zinc homeostasis (Leung et al., 2008). Light microscopic analysis of 15- to 30-micron retinal sections has revealed a rich band of zinc ion transporter 3 (ZnT-3) protein in the region of the outer limiting

membrane and photoreceptor inner segments. ZnT-3 reactivity has also been shown to be present in the outer plexiform, inner nuclear, inner plexiform, and ganglion cell layers. ZnT-3 is the output type zinc ion transporter, located in the retina, brain, testis, and it is responsible for zinc transport from the tissue cells to the extracellular medium (Redenti and Chappell, 2004). ZnT-3 has been investigated with regard to its specific expression as a brain zinc ion transporter, but little studied in the retina.

This study examined in rats the effect of the low-zinc diet for different lengths of time on serum content of zinc and vitamin A and ZnT-3 mRNA expression in the retina, exploring how a low-zinc diet influences ZnT-3 transcriptional regulation and zinc enzyme CA2 and CA14 mRNA expression in the retina.

MATERIAL AND METHODS

Animals

Thirty-six male mice, 36 weeks old, were purchased from Shanghai Slac Laboratory Animal Co. Ltd. Animals were housed under controlled conditions of light (12-h light and 12-h darkness). After 3-4 days under these conditions, animals were randomly divided into three groups: 1) fed for 2 weeks (the first group); 2) fed for 4 weeks (the second group); 3) fed for 6 weeks (the third group). Every group was randomly divided into two subgroups, which were given a normal-zinc diet (called "N subgroup") and a low-zinc diet (called "L subgroup"). The commercial diet containing a normal amount of zinc (>30 mg Zn/kg) was supplied to the N subgroup; deionized water and the synthetic diet (<10 mg Zn/kg) prepared from AIN-76™ synthetic formulation was given to the mice in the L subgroup. The mice in the L subgroup of the third group received normal diet on the fourth week. Mice received food and water *ad libitum*. The zinc content of the low-zinc diet was 4.34 mg Zn/kg as measured by atomic absorption spectrophotometry.

Tissue sampling

After animals were anesthetized with 1% chloral hydrate, the eyeball was enucleated and placed on ice. The retina was then completely and carefully detached with ophthalmic scissors and forceps under a stereomicroscope as quickly as possible. The detached retina was temporarily stored in liquid nitrogen and then moved to a refrigerator at 37°C. A 4-mL blood sample was drawn from the heart, and the serum was separated and stored at low temperature for later use.

Serum zinc and vitamin A levels

Vitamin A in serum was determined by high-performance liquid chromatography (LC-10A SPA-10A). Serum zinc content was measured by flame atomic absorption spectrometry (AA-6501F), which always analyzes the contents of copper in WS/T93-1996 serum.

Real-time PCR

Total RNA was extracted from tissues using TRIzol. Two microliters of RNA was di-

luted 50 times with EDPC and quantified with a ultraviolet spectrophotometer. cDNA was prepared from 1 μg total RNA by reverse transcription. PCR was carried out using 1.5 μL cDNA target. After PCR, 8-μL products were analyzed on a 1.5% agarose gel. By analyzing the gray scale of the internal control GAPDH and the products of the interest with gel imaging system and the image analysis software (UVP Inc., USA), the OD values of the internal control and each product were obtained. The relative expression amount of the products was determined according to the amount of GAPDH. The primers and the optimal annealing temperature for real-time PCR are shown in Table 1.

Table 1. Primers and the optimal annealing temperature for real-time PCR.

mRNA	Primer sequence	bp	Annealing temperature (°C)
GAPDH	Sense: 5'-ACCACAGTCCATGCCATCAC-3'	468	58.5
	Antisense: 5'-TCCACCACCCTGTTGCTGTA-3'		
ZnT-3	Sense: 5'-TCTCTGACCACTCCGCCCCT-3'	396	63.0
	Antisense: 5'-GCCTGCCACCATGCTACCAC-3'		
CA2	Sense: 5'-CTCTCTGACCACTCCGCCCC-3'	404	62.3
	Antisense: 5'-CTCTCTGACCACTCCGCCCC-3'		
CA14	Sense: 5'-CATCTTTGCTTCTTTCATCC-3'	140	56.2
	Antisense: 5'-CATCTTTGCTTCTTTCATCC-3'		

Statistical methods

The SPSS 11.5 software was used for analysis. An independent sample *t*-test was carried out after a homogeneity test of the variances of measurement data between groups. The significance level was set at 0.05.

RESULTS

Dietary zinc deficiency and serum zinc and vitamin A content

Serum zinc and vitamin A levels in G1 of the low-zinc diet for 2 weeks were significantly lower than those in G1 of the normal diet group. Serum zinc and vitamin A levels in G2 of the low-zinc diet for 4 weeks were lower than those in G1 of the normal diet group, but without significant difference. Serum zinc and vitamin A levels in G3 of the normal-zinc diet for 2 weeks were not significantly different from those in G3 of the normal diet group (Table 2).

Table 2. Comparison of serum zinc and vitamin A in two sets.

Serum content	Subgroup	G1	G2	G3
		2 (weeks)	4 (weeks)	6 (weeks)
Zinc (mg/L)	N	1.26 ± 0.09	1.04 ± 0.14	1.14 ± 0.05
	L	0.77 ± 0.95*	0.87 ± 0.10	1.24 ± 0.09
Vit A (μg/L)	N	201.79 ± 11.46	180.99 ± 14.53	165.21 ± 15.90
	L	145.0 ± 9.66*	141.14 ± 18.71	138.55 ± 20.47

Data are reported as means ± SD. Low-zinc subgroup (L) compared with the normal subgroup (N), *$P < 0.05$.

Dietary zinc deficiency and retinal ZnT-3 mRNA levels in CA2 and CA14

ZnT-3, CA2 and CA14 mRNA expression levels in rat retina in G2 of low-zinc diet for 4 weeks were significantly lower than in the normal control group. No significant difference was found for ZnT-3, CA2 and CA14 mRNA expression in rat retina between the low-zinc diet subgroup and the normal-diet subgroup in G1 and G3 (Table 3).

Table 3. Comparison of the mRNA expression in retina product of rats in two sets.

		G1 2 (weeks)	G2 4 (weeks)	G3 6 (weeks)
ZnT-3	N	1.36 ± 0.21	2.18 ± 0.18	1.91 ± 0.27
	L	1.70 ± 0.19	0.80 ± 0.17*	2.32 ± 0.17
CA2	N	1.60 ± 0.79	2.05 ± 0.23	1.67 ± 0.20
	L	1.32 ± 0.90	1.10 ± 0.20*	1.67 ± 0.14
CA14	N	0.86 ± 0.09	0.86 ± 0.11	0.83 ± 0.15
	L	0.78 ± 0.06	0.48 ± 0.04*	0.91 ± 0.12

Data are reported as means ± SD. Low-zinc subgroup (L) compared with the normal subgroup (N), *P < 0.05.

Changes in ZnT-3, CA2, CA14 mRNA levels detected by RT-PCR

There were changes in ZnT-3, CA2 and CA14 mRNA expression in rat retina. With low-zinc diet for 2 weeks, ZnT-3 mRNA expression was increased in the retina and CA2 and CA14 mRNA levels decreased, but no significant differences compared with the normal group. With low-zinc diet for 4 weeks, ZnT-3, CA2 and CA14 mRNA expression in the retina decreased significantly compared with the normal group. With normal-zinc diet for 2 weeks, ZnT-3, CA2 and CA14 mRNA expression in the retina was not significantly different compared with the normal group (Figures 1-4).
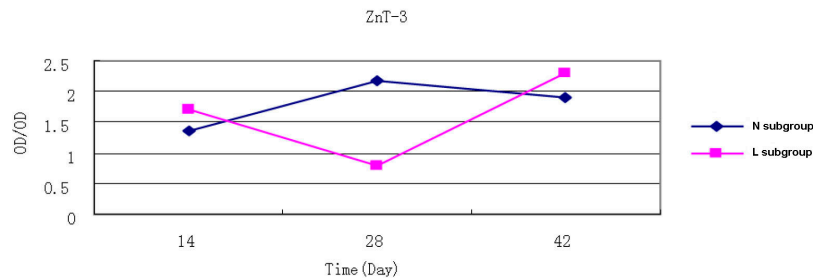

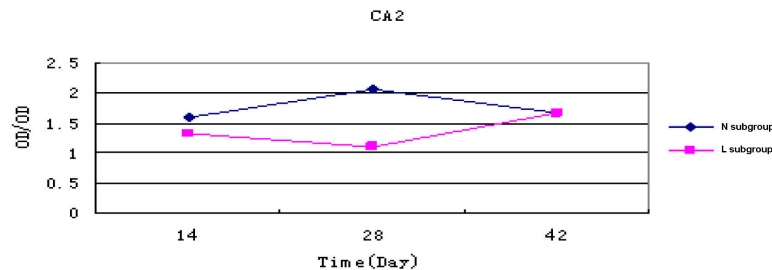**Figure 1.** Statistic graph of ZnT-3 mRNA expression of rat retina.

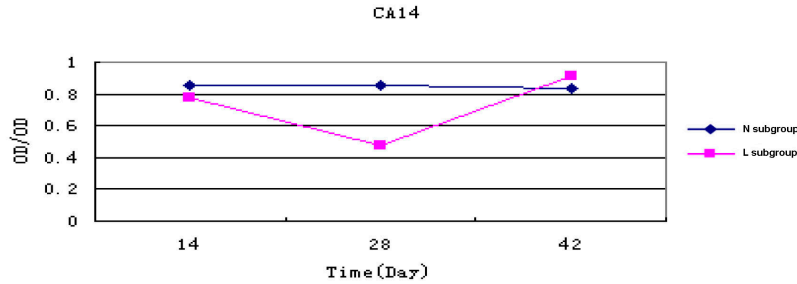**Figure 2.** Statistic graph of CA2 mRNA expression of rat retina.

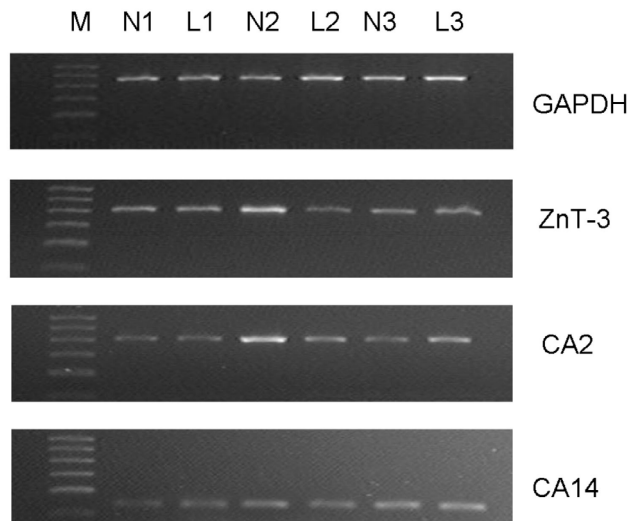**Figure 3.** Statistic graph of CA14 mRNA expression of rat retina.

Figure 4. RT-PCR result of ZnT-3, CA2 and CA14 mRNA expression of rat retina. M = marker; N = normal-zinc diet subgroup; L = low-zing diet subgroup.

DISCUSSION

Zinc deficiency in rats dramatically affects ocular development. Grahn reported significant retinal dysplasia and depression of the electroretinograms in rats born to dams that were marginally zinc- and taurine-deficient throughout gestation and postnatal life until 7.5 to 8.5 weeks of age (Grahn et al., 2001). They found that serum Zn decreased in Zn-depleted rats during the depletion phase and was lower in Zn-depleted and marginal-Zn rats than in the Zn-replete controls throughout the study. In their study, dietary repletion of Zn normalized serum Zn. Similarly, Zn depletion reduced bone Zn compared with Zn-replete controls. Femur Zn continued to decrease in marginal-Zn rats throughout the study and did not return to Zn-replete control levels during the repletion phase, showing that marginal-Zn rats continued to deplete bone stores of Zn throughout the experiment and that a 2-month repletion phase was too short to fully refill Zn stores in the bone compartment after long-term marginal-Zn supply (Reinhold et al., 2009). Studies reveal that retinal zinc concentrations are conserved in marginal zinc

deficiency in the rat despite significant decreases in serum, liver and bone zinc concentrations (Grahn et al., 2001).

In mammalian tissues, cytoplasmic Zn^{2+} concentration is maintained within a narrow range in the cells. Cells homeostatically adjust to Zn^{2+} excess by sequestering the metal ion in cytoplasmic vesicles or by increasing its efflux, and under conditions of zinc deficiency, Zn^{2+} influx is increased. These adjustments to zinc distribution and homeostasis involve complex cellular mechanisms that rely on many integral membrane transporters and metallochaperones to maintain a strict balance of intracellular zinc (Leung et al., 2008). ZnT transporters are efflux transporters that reduce cytoplasmic Zn^{2+} concentrations by promoting zinc efflux from the cytoplasm to the extracellular compartment or into intracellular vesicles. ZIP transporters, on the other hand, are the influx transporters that mediate Zn^{2+} uptake into the cytoplasm from extracellular or vesicular sources (Cousins et al., 2006).

Leung et al. (2008) found that the RPE likely comprises critical cell types that regulate retinal zinc ion balance, and that are related to the control of retinal dysfunction under conditions of pathological retinal zinc levels. RPE cells through the role of zinc transporters in the retina zinc pool has not been studied.

Redenti and Chappell (2004) examined the distribution of the ZnT-3 protein in the light-adapted mouse retina using immunohistochemical techniques, and revealed a rich band of the ZnT-3 protein in the region of the outer limiting membrane and photoreceptor inner segments. ZnT-3 reactivity was also present in the outer plexiform, inner nuclear, inner plexiform, and ganglion cell layers. The outer nuclear layer and photoreceptor outer segments did not exhibit ZnT-3 immunoreactivity. In the light-adapted murine retina, ZnT-3 appears to localize in regions that have been found reactive for ionic zinc.

Redenti and Chappell (2007) revealed an unexpectedly high concentration of this transporter protein in the outer limiting membrane region of the murine retina, suggesting that Müller cells utilize ZnT-3 to regulate retinal zinc homeostasis and that this role is important to mitochondrial function in the photoreceptor inner segments. ZnT-3 plays an important role in regulating retinal zinc ion balance and maintaining visual function.

The results of this study showed that a significant decrease in serum zinc levels and ZnT-3 mRNA expression in the retina with no significant increase compared to the normal group after zinc-deficient diet for 2 weeks, indicated that zinc outflow in the retinal cells did not significantly increase. Serum zinc level was lower than in the normal control group, but there was no significant difference after 4 weeks of low-zinc diet. While ZnT-3 mRNA expression in retina was significantly decreased, it showed that main body zinc pool, such as bone and liver zinc outflow, increased in blood, and serum zinc levels does not reflect the status of zinc in the body cells.

Many zinc-containing enzymes are present in the visual organization of cells, especially the retina and choroid. CA2 is involved in the visual development process (Adijanto et al., 2009). CA2 and CA14 can absorb subretinal fluid in macular edema. The CA14 can speed up the removal of CO_2 from the neural retina and regulate the function of photoreceptors. Retinal zinc deficiency influences visual function by affecting the activity of its zinc-containing enzymes (Adijanto et al., 2009; Casey et al., 2009).

This study showed that after a low-zinc diet for 2 weeks, ZnT-3 mRNA expression was increased in the retina, and CA2 and CA14 mRNA levels decreased but not significantly compared with the normal group. After a low-zinc diet for 4 weeks, ZnT-3, CA2 and CA14

mRNA expression in the retina significantly decreased compared with the normal group. To maintain zinc content in the retina and CA activity, ZnT-3 expression was lowered to reduce the zinc loss from retinal tissue. With resumed normal-zinc diet for 2 weeks, ZnT-3, CA2 and CA14 mRNA expression in the retina did not significantly differ compared with the normal group. This indicated that ZnT-3 expression in the retina is a reversible process after low-zinc diet; sand retinal reserved a fine adjustment of the function of the level of zinc content. The regulation of CA2 and CA14 expression was related to the zinc content of retinal cells, but not completely consistent with changes in serum zinc levels.

Vitamin A is important in maintaining normal vision substances and plays an important role in visual development. The study showed that there were synergies between the zinc and vitamin A. Experimental results showed that zinc deficiency caused by the reduction of the level of vitamin A in rats, consistent with the changes in serum zinc content. That described body absorption of vitamin A may be the influence of zinc in the body. Low zinc can reduce the absorption of vitamin A; it was consistent with Christian and West Jr. (1998) and Kelleher and Lönnerdal (2002) research on the interaction between the zinc and vitamin A. That indicated zinc deficiency through indirectly affect the development of visual function caused by vitamin A decline.

This study shows that retinal cells at the transcriptional level can be adjusted by changing the way of the ZnT-3 mRNA expression in the zinc-deficiency conditions, reduce the zinc outflow of retinal tissue, and maintain the stability of the zinc content of the retinal tissue. The decrease in CA2 and CA14 mRNA expression is likely to affect normal visual function.

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