Effects of Intracellular Zinc Chelator In Vivo on Taurine Transport, Taurine Concentrations and Taurine Transporter in Rat Retina Cells

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Abstract

Taurine and zinc, highly concentrated in the retina, have neurotrophic effects in the central nervous system. Zinc modulates Na+/Cl- dependent transporters, such as those of dopamine and taurine (TAUT) in vitro, although there is no evidence of in vivo effects of zinc. The objectives of the present study were to evaluate the consequences of zinc deficiency on taurine levels and transport, and mRNA levels of TAUT in rat retina. Various concentrations of the intracellular zinc chelator, N,N,N,N-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN), dissolved in dimethylsulfoxide, were administered intraocular for dose selection: 1, 2.5 and 5 nM (12.5, 31.25 and 62.5 nM final concentrations). Dilution in the eye is approximately 25 times. It is taking into account the volume of the eye, 12.5 µl). Retinas were dissected 3, 5 and 10 days later. Zinc was determined by spectrophotometry. TPEN administration, 5 nM, decreased zinc in 67% at 5 days. Taurine levels, determined by high performance liquid chromatography with fluorescence detection, were 65.96 ± 4.73 nmol/mg protein in tissue, 44.34 ± 5.55 in isolated cells, and in 6.63 ± 1.12 in cell membranes. Capacity of taurine transport, using [3H]taurine, was decreased in 38% and affinity was increased in 50% after TPEN. mRNA levels of TAUT, by RT-PCR, were diminished in 50% by the chelator. Optimal concentrations of zinc are necessary for the equilibrium of taurine system in the retina, which involves concentrations of taurine, taurine transport and TAUT mRNA levels.

Keywords: Retina; Taurine; Taurine transporter mRNA; Zinc

Abbreviations: ICP: Inductively Coupled Plasma Emission Spectrophotometry; io: Intraocular; NMG: N-methyl-glucamine; RPE: Retinal Pigment Epithelium; TAUT: Taurine Transporter; TPEN: N,N,N,N-tetrakis-(2-pyridylmethyl) Ethylenediamine

Introduction

Taurine possesses neuroprotective and neurotrophic properties in the central nervous system (CNS) during development and regeneration, and its levels are high in the eye, particularly in the retina, ranging from 50 to 80 mM [1]. The system of taurine in the retina has been studied, such as synthesis, transport, storage [2-9] and cell localization [10-14]. In addition, zinc has been recognized as an essential element, crucial for development and function of CNS, abundant in plants and animals, second after iron [15]. Zinc content in the retina is also high, reaching concentrations up to 100 µg/g [16]. Zinc deficiency in humans has been linked to an abnormal adaptation to darkness, cataracts and blindness, as well as macular degeneration, due to decrease of antioxidants in the retina [15,17]. The role of zinc in the eye is associated with its coexistence with other molecules such as the amino acid taurine [16].

As pointed above, taurine and zinc possess similar functions in the CNS, related to protection and regeneration. Despite quoted experimental approaches, there is no evidence about the effects of zinc on retinal taurine system of mammals, in regard to concentration, transport activity and relative levels of mRNA of transporter. Zinc regulates several Na+/Cl- dependent neurotransmitter transporters, such as that of dopamine [18], norepinephrine [19], serotonin [20], and taurine (TAUT) [8-9,12,21].

We have previously shown that intermediate concentrations of zinc obtained by the intracellular chelator, N,N,N,N-tetrakis-(2-pyridylmethyl) ethylenediamine (TPEN) ex vivo significantly decreased [3H]taurine transport [21]. In addition, the reduction of zinc by TPEN in the retina, significantly decreased TAUT in all layers, as detected by immunohistochemistry, except in photoreceptors, indicating that the presence of zinc is necessary for maintenance of the transporter in the retina [22].

The study of possible effects of zinc on taurine system in the retina is relatively novel and of great interest for better understanding the interactions of taurine and zinc in this structure. Since both molecules have physiopathological relations, this work examined the effects caused by chelating zinc by injection of TPEN into the eye on taurine levels, taurine transport and TAUT mRNA in rat retina.

Materials and Methods

Animals

Male Sprague-Dawley rats (150-200 g) from the animal housing at Instituto Venezolano de Investigaciones Científicas had an adaptation period of at least 48 hours in the experimental room of the Laboratory, food and water provided ad libitum. The animals were decapitated between 8:00 and 10:00 am, and the eyes were extracted from orbit. Handling of animals was conducted following the standards of animal bioethics [23] and was approved by the Bioethics Committee for Animal Research of the Institute.

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Treatment with the intracellular zinc chelator N,N,N,N-tetrakis-(2-pyridylmethyl) ethylenediamine in vivo

Various concentrations of intracellular zinc chelator TPEN, 1, 2.5 and 5 nM, were administered for different periods of time 3, 5 and 10 days, for selecting the dose to be used according to zinc levels reduction [9]. Three experimental groups of rats were included:

1) Intracocular administration (io) of 1, 2.5 or 5 nM of TPEN, final concentrations 12.5, 31.25 and 62.5 nM (dilution in the eye is approximately 25 times. It is taking into account the volume of the eye, 12.5 µl), 2 µl of saline and dimethyl sulfoxide (DMSO, 0.001%).

2) Administration (io) of 2 µl of DMSO 0.001% final concentration (vehicle, control group).

3) Needle puncture (sham intervention).

Isolation of rat retinal cells

Retina was dissected and cells were separated with 0.25% trypsin in Locke buffer (500 µl), composed (in mM) of 154 NaCl, 2.7 KCl, 2.1 K₂HPO₄, 0.95 KH₂PO₄, 2.7 sucrose and 2.5 HEPES, at 37°C for 10 min, followed by mechanical further separation with Pasteur pipette. The cells were washed with phosphate saline buffer Na `-K` (PBS) 0.1 M pH 7.4, centrifuged for 10 min at 2,000 rpm (300 g), and counted in Neubauer chamber. Integrity of membrane was determined by 50% Trypan blue exclusion (>96%) [21].

Determination of total zinc

The tissue was homogenized in 320 µl of deionized distilled water, 20 µl were collected for protein determination by bichinonic acid (BCA) assay [24], and the remainder was used for determination of total zinc. Tissue samples were dried, suspended in 1 ml of concentrated HNO₃, heated for 6 h at 60°C and made up to 10 ml with deionized water. Inductively coupled plasma emission spectrometry (ICP-AES) was performed in a Perkin–Elmer Model Optima 3000 equipped with a U-5000AT ultrasonic nebulizer, a standard demountable-type quartz plasma torch and alumina injection (1.5 mm internal diameter). A ten-roller peristaltic pump was used to supply the gas with the sample solution. The atomic line for zinc was 213.856 nm [9,25,26]. After verified reduction of zinc levels, 5 nM of TPEN, 5 days after io injection was chosen for all the experiments.

Determination of taurine

Taurine was determined in rat retina: tissue, isolated cells and cell membranes, and after TPEN injection in isolated cells. HPLC with fluorescent detection was employed by a modified method [26]. The system consisted of a Waters 2690 Separation System and a Shimadzu UV-2600 with a Waters 996 PDA detector. Separation was done with a Supelco LC-18 column 4.6 × 100 mm, 5 µm. The samples was resuspended and homogenized in 50 µl of 20% sulfoalicylic acid and 300 µl of 0.4 M potassium borate buffer pH 10.4 and the aliquots were subjected to protein analysis by BCA assay [24]. Centrifugation of samples was carried out at 38,000 g for 20 min at 4°C. Derivatization was performed by addition of 200 µl of the following mix: 25 mg ortho-phthaldehyde, 500 µl methanol, 25 µl β-mercaptopethanol (1 g/ml), and 4.5 ml 0.4 M potassium borate buffer of pH 10.4. Aliquots of the derivatization preparation were injected into the chromatographic system. Taurine was quantified by the method of the external standard and expressed in nmol/mg of protein using the program Millenium (Waters, MA, USA) [25].
2. \( V_{\text{max}} \) and \( K_t \) of taurine transport were calculated either by Lineweaver-Burk plots or curvilinear analysis. Values of \( p < 0.05 \) were considered for statistically significant.

**Results**

**Zinc levels after io injection of intracellular zinc chelator**

There was no significant difference between the groups receiving the vehicle, DMSO or the needle puncture, thus control group was that with injection of DMSO was observed that the concentration of zinc in the control group was 93.75 ± 10.66 µg/mg of protein. Zinc levels were significantly decreased to 37.66 ± 3.4 µg/mg of protein at 5 days after the io administration of TPEN in a concentration of 5 nM with respect to DMSO (\( F_{1,20} = 9.39 \) \( P < 0.05 \) (Figure 1A). At 3 and 10 days, TPEN did not produce a significant decrease in zinc levels in the retinas of the eyes treated with different concentrations of the chelators compared to DMSO (\( F_{1,20} = 1.79 \) \( P > 0.05 \) and \( F_{1,20} = 1.401 \) \( P > 0.05 \), respectively) (Figures 1B and 1C). The treatment with TPEN did not modify Ca\(^{2+}\), Mg\(^{2+}\), Fe\(^{3+}\), and Na\(^{+}\) levels in the retina.

**Taurine levels after injection of intracellular zinc chelator**

Taurine levels were 65.96 ± 4.73 nmoles/mg of protein in retinal tissue, 44.34 ± 5.55 in isolated cells, and 6.63 ± 1.12 in cell membranes. The concentration of taurine in isolated cells from DMSO was 48.07 ± 8.84 nmoles/mg of protein and was significantly reduced in 66.62% (16.05 ± 2.37 nmoles/mg of protein) at 5 days after the io administration of 5 nM TPEN (Figure 2).

**Taurine transport after injection of intracellular zinc chelator**

The analysis of saturation experiments of \([3H]\)taurine transport was best fitted to a two-site model (Figure 3A). In the presence of increasing concentration of \([3H]\)taurine covering the high affinity range, there was best fit to a one-site model (Figure 3B). The value of \( V_{\text{max}} \) was 9.77 ± 1.46 fmol/10\(^6\) cells and for \( K_t = 0.08 ± 0.04 \) µM (Figure 4). 5 Days after the io administration of 5 nM TPEN, taurine transport decreased respect to control group (Figure 5). There was a significant reduction of 38.24% in the capacity and significant increase of 50% in the affinity for taurine (Table 1).

**Levels of mRNA of taurine transporter after injection of intracellular zinc chelator**

The levels of mRNA TAUT in the DMSO group were 3.83 ± 0.53 equivalents. There was a significant decrease in relative levels of mRNA TAUT after treatment with TPEN: 2.06 ± 0.39 (Figures 6 and 7).

**Discussion**

**Zinc levels and effect of intracellular zinc chelator**

Intracellular and extracellular levels of zinc are critical for the proper functioning of the nervous system [36]. Total content of zinc in plasma is about 100 µg/100 ml, which variations depending on age, gender, pregnancy and time of day [15,37]. The intracellular concentration of total zinc is around 100 µM, and in cytoplasm, between 3 and 100 nM, facing extracellular zinc below 500 nM [38]. Zinc, 1 to 50 µM, increases neuronal excitability and greater than 100 µM is inhibitory [38]. During intense activity in the hippocampus, zinc gets to 300 µM in cytoplasm [38] and up to 20 mM in vesicles [37].
These compartmental differences points to fine regulation of zinc levels due to proper activity of their transporters. The amount of zinc in some of the tissues of the eye is very high and in some animals could be twice the concentrations reported in other tissues [16]. Moreover, these authors indicate that the amount of zinc varies with age and type of ocular tissue, with highest concentration in the retina. In the human retina, zinc concentration is 100 µg/g [16,39], also present in cornea, vitreous humor, lens, iris and ciliary body [39]. The concentration of cellular zinc requires establishment of discrete, active cellular gradients. The cellular distribution of zinc into organelles is precisely managed to compartment or into intracellular vesicles. ZIP transporters, on the other hand, are the influx transporters that mediate Zn2+ uptake into the cytoplasm from extracellular or vesicular sources [41].

Using various concentrations of the intracellular zinc chelator TPEN [9] and different periods of treatment, tested for selecting the dose to be used in further experiments (see Materials and Methods), we detected the reduction of zinc at specific conditions. The use of zinc chelators is an useful alternative for restricting its levels in the cells. Moreover, the advantage of local injections allows exploring an area of interest and interpretation of the results could be less confusing. Intracellular zinc chelator, TPEN, among others, is one of the most widely used, for effectiveness and lack of toxicity at the administered doses, in addition, it possesses high affinity for zinc (4 x 10^{-11} M) [42-44]. On the other hand, it has been reported that zinc deficient diets have little effect on the reduction of zinc in tissues such as the brain, for instance, decrease of 30% occurs after of 3 months in the hippocampus [43]. Thus, the determination of total zinc by ICP showed that this chelator produced a significant decrease of zinc in retina, and that had greater effect on the reduction of zinc at nM concentrations compared to the extracellular zinc chelator diethylenetriaminepentaacetic acid (DTPA) [25]. Intracellular chelators, such as TPEN, are usually more effective in reducing the metal and lower concentrations are required to cause the effect, as it has been previously shown in ex vivo studies of taurine transport [21] and with the in vivo results of this study. Depending on the amount of both, zinc released and chelator present, exogenous chelators might have either a neuroprotective or neurotoxic effect based on their ability to lower endogenous zinc levels [43,45].

Depending on the amount of both, zinc released and chelator present, exogenous chelators might have either a neuroprotective or neurotoxic effect based on their ability to lower endogenous zinc levels [43,45]. The difference between the compartmental action, intra- or extracellular, for membrane-permeant and impermeant chelators, respectively, containing different zinc-binding ligands [38]. These facts might contribute to the observed differences. After studying several days of treatment with variable doses of TPEN, 5 nM reduced the concentration of zinc in the rat retina 5 days after injection. It was observed that the reduction of the levels of zinc in the retina was time dependent and the concentration of the chelator. Since the at 3 and 10 days TPEN did not produce a significant decrease in zinc levels in the retinas of the eyes treated with different concentrations of the chelators compared to DMSO. Unlike, at 5 days after the io administration of TPEN in a concentration of 5 nM, zinc levels were significantly decreased as shown in Figure 1B. This treatment produced significant differences in various parameters of taurine as shown. These results suggest that zinc in the retina is associated with other molecules such as taurine, moreover, might be related to the formation of taurine-zinc complexes in cell membranes of retina.

Figure 3: Curve of the saturation of [3H]taurine transport into rat retinal cells determined by presence of concentrations of [3H]taurine from 50 to 500 nM. Data were best fitted to: A) a rectangular hyperbole $R^2 = 0.97$, $n = 5$ and B) Lineweaver-Burk analysis of the same results of Figure A $R^2 = 0.997$, $n = 4$. Each value is the mean ± SEM.

Figure 4: Curve of saturation of [3H]taurine transport. Was performed in the presence of different concentrations of [3H]taurine of 15 to 135 nM, with a time of preincubation of 5 min and 25 s of incubation at 37 °C. Fit hyperbola 1 site. $R^2 = 0.92$, $n = 4$. $V_{max} = 9.77 ± 1.46 \text{ fmol/10}^6\text{ cells$ and K}_I = 0.08 ± 0.04 \mu M$. $V_{max}$ and $V_{max}$ were determined by the method of double reciprocal of Lineweaver-Burk. Each value is the mean ± SEM, $n = 4$. 

<insert image of Figure 3 and Figure 4 here>
Levels of taurine, taurine transport, mRNA of taurine transporter and effect of intracellular zinc chelator in retina

The presence of zinc in the retina and its interaction with taurine contribute to the functional integrity of this structure [16,39]. Stockner et al. [46] demonstrated that taurine and zinc are located on the periphery of the rods in the retina of cat, and that reducing the concentration of taurine decreases zinc levels in rods, since both molecules form complexes that are inserted into cell membrane for stabilization. Moreover, it has been shown that there is an increased in rat urine excretion of taurine due to zinc deficient diet [47]. Taurine and zinc protect photoreceptors from peroxidation in the retina of frog exposed to ferrous sulfate, and addition of taurine or zinc alone has no effect, although the conjoint supply of both molecules is protective [48]. Gottschal-Pass et al. [49] reported that taurine and zinc interact during development of the retina, they used light microscopy techniques and oscillatory potentials. These authors note that in rats fed zinc and taurine deficient diets occurs dysplasia in the retina, with photoreceptor degeneration and alterations in the oscillatory potentials.

There is no evidence about the effects of zinc on retinal taurine system of mammals, in regard to concentration, transport activity and relative levels of mRNA of transporter. It is known that zinc regulates several Na+/Cl- dependent neurotransmitter transporters, such as that of dopamine [18,50], norepinephrine [19], serotonin [20], and taurine [8,9,12,21]. Dopamine, serotonin and taurine transporter belongs to the family of Na+/Cl- dependent transporters.

Taurine transport in isolated cells of the retina of goldfish and rat is mediated by two saturable components, of high and low affinity [1,2], as we observed in the present study.

Since low levels of zinc, caused by TPEN treatment in vivo, produced significant decrease in taurine levels and transport, zinc might bind some site of TAUT that affect union or translocation of taurine, or possibly the formation of taurine-zinc complexes could be involved in maintaining the functional stability of the retina. It has been reported that zinc is a noncompetitive inhibitor of dopamine transporter and these effects occur due to binding to a particular transporter site affecting translocation of dopamine [51]. It has been shown that zinc increases serotonin uptake concentration-dependent manner in sections of the corpus callosum in adult rats [20]. It is not known if TAUT has residues of His in any of their domains, which may be the recognition site of the metal, as demonstrated for serotonin [52] and dopamine transporters [18,53]. By mutagenesis and molecular models, binding sites of zinc to His and Cys residues has been identified in serotonin transporter, which are located near the extracellular ends of the transmembrane helices I and III [52]. The tertiary structure of dopamine transporter is modified, involving His193, His375 and Glu396 residues, which are close together [50]. Stockner et al. [53], by mutagenesis and structural changes, confirmed the presence of these residues interacting with zinc. Changes of transporter structure could influence its sensitivity to other regulatory processes, such as phosphorylation. Zinc has a direct inhibitory effect on glutamate transporter in cells of salamander retina [54]. In fact, it has been demonstrated that zinc has a biphasic effect on taurine transport ex vivo, stimulating it at low concentrations and inhibiting at high [21]. In the presence of 30 and 40 µM there was a significant increase of the transport, concentrations of 100, 150 and 200 µM of ZnSO4 reduced it. [3H]Taurine transport was significantly reduced with 20 µM TPEN, incubating the cells with TPEN plus zinc, 100 µM, did not significantly affect it, and the presence of zinc in the incubation medium significantly decreased it. This evidence indicates that zinc affects various transport systems, including that of taurine in retina, as shown by our findings.

The genomic effects of zinc mediated by transcription factors could also be of relevance, including identified genes in intestine, thymus and monocytes involved in signal transduction, immune response, stress reactions, growth and energy utilization, which are regulated by proper
concentrations of zinc trough its transporters [55]. Significant changes in mRNA levels of TAUT in isolated rat retinal cells were observed 5 days after the io administration of TPEN, these results suggest that variations in taurine levels and transport could be due to changes in the relative levels of mRNA of TAUT. It has been suggested that one of the mechanisms regulating TAUT is by transcription factors, the promoter of TAUT has multiple binding sites for different transcription factors, which are: the tumor suppressor gene (P53), the transcriptional activator, V-myb avian myeloblastosis viral oncogene homolog, activator protein-1, Wills’ I tumor suppressor gene Wills’ I (WT1), and protein specificity I (SP1) [3,56]. P53 requires zinc molecules to maintain its structure, sequence-specific binding to DNA depends on the metal [57,58]. WT1 and SP1 are transcription factors which contain a zinc finger motif through which bind directly to DNA and promote transcription [59,60]. Recently, it has been suggested that SP1 is necessary to activate the promoter of TAUT [56]. It could be that the decrease in zinc caused by TPEN modified transport activity, concentrations of taurine and levels of TAUT mRNA by effects on transcription factors regulated by zinc.

Conflict of Interests

There is no conflict of interest. The authors have non-financial competing interests in an exclusive academic way. A. Márquez is a PhD, who performed all experiments and writing. M. Quintal was in charge of zinc determinations. M. Urbina was participated in transport experiments and PCR assays. F. Obregón was in charge of taurine determinations. L. Lima, Chairperson and Tutor, provided with original idea, financial support, supervision of each experiments, analyzing results, discussion and writing.

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