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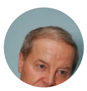
Maksim Zakhartsev
Hohenheim University



V. P. Chelomin
V.I. Il'ichev Pacific Oceanological Ins…

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In vitro effect of copper ions on transbilayer distribution of aminophospholipids in synaptosomal membrane of walleye pollock (*Theragra chalcogramma*)

A.V. Kurilenko, M.V. Zakhartsev *, V.P. Chelomin

Pacific Oceanological Institute, Russian Academy of Sciences, Vladivostok 690041, Russia

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Abstract

Effect of copper ions on lipid matrix organization of synaptosomal membrane of the marine fish *Theragra chalcogramma* was investigated. It was demonstrated that interaction of copper ions with these membrane stimulated the process of lipid peroxidation and caused changes in the transbilayer distribution of aminophospholipids. Accessibility of phosphatidylethanolamine was increased more than twice, and of phosphatidylserine more than ten times, that can be explained by changes in asymmetrical structure of lipid matrix of synaptosomal membrane. We suggested, that the main mechanism of copper-stimulated damage in transbilayer organization of the membrane is oxidation of membrane protein sulfhydryl groups. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Copper; Phosholipids; Membrane asymmetry; Sulfhydryl groups; Lipid peroxidation; *Theragra chalcogramma*

1. Introduction

Even the subtlest changes in morphology or function must be preceded by biochemical changes. Heavy metals, in particular copper, have been reported to have adverse an influence on cells of different hydrobionts, and a number of biochemical shifts were found (Viarengo, 1985; Nor, 1987; Gould et al., 1988; Arasu and Reddy, 1995). Nevertheless, there are still many obscurities in our notions about the destructive processes that take place due to accumulation of copper ions.

Many diverse metabolic and structural changes associated with metal toxicity in cells probably result from a cascading process, which originates from a primary lesion, e.g. biomembranes. The prime target sites for heavy metal toxicity are surface membranes (Christie and Costa, 1984) possibly due to their exposed location and chemical reactivity. Biological membranes are self-assembling bioorganic systems and are responsible for most of the vital functions of cells and subcellular organelles. Therefore, knowledge of metal-induced changes in the membrane at the

* Corresponding author. Present address: Department of Biology, Ecophysiology and Biochemistry, University of Antwerp, RUCA, 2020 Antwerp, Belgium. Tel.: +32-3-2180-342; fax: +32-3-2180-497.

E-mail address: zakhar@ruca.ua.ac.be (M.V. Zakhartsev).

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molecular level is essential for understanding the molecular basis of heavy metal toxicity in general.

The asymmetric distribution of membrane phospholipids between the outer and inner monolayers is one of the fundamental attributes of biological membranes. The present study was undertaken to evaluate the possibility of copper-stimulated damage of the transbilayer organization of phospholipids (aminophospholipids, e.g. phosphatidylethanolamine and phosphatidylserine) in the synaptosomal membranes of the marine fish *Theragra chalcogramma*.

2. Materials and methods

2.1. Animals

The experimental research was carried out on adult walleye pollock, *T. chalcogramma* (30–35 cm length; $n = 10–12$ fishes per capture), which were caught by trawling (amount of captures are $n = 6$) in the same location in south part of Peter the Great Bay (the Sea of Japan) between August and September of 1995. The fishes were killed right after catch by decapitation, and brains were pooled and kept on ice (+1 to +4 °C) in a thermostated container until use (no more than 12 h).

2.2. Membrane preparation and exposure procedure

The synaptosomal membranes from pooled fishes' brains were isolated according to Hajos (1975). The membrane fraction was exposed to 100 μg/l copper (as $CuSO_4 \times 5H_2O$) in a buffer containing of 150 mM NaCl, 50 mM Tris–HCl (pH 7.5) for 90 min at 20 °C. The concentration of copper 100 μg/l was chosen as a concentration, which certainly has effect on biochemical level and, in the same time, not too high from ecological point of view (Nor, 1987). A control membrane fraction was incubated in the same conditions, however, without copper ions. After 90 min of Cu-treatment EDTA was added to a final concentration of 10 mM, then membranes were precipitated by centrifugation for 15 min at $10\,000 \times g$. The membrane pellet was washed three times in the same copper free buffer. The final pellet of synaptosomal membranes was resuspended in 150 mM NaCl, 50 mM Tris–HCl (pH 7.5) buffer and used for the experiments.

2.3. Labeling of membrane lipids and lipid analysis

Transbilayer distribution of membrane aminophospholipids was evaluated by means of a kinetic technique with non-penetrating reagent 2,4,6-trinitrobenzenesulfonate (TNBS). The fractions of the synaptosomal membrane were treated with 2.5 mM of TNBS in medium 150 mM NaCl, 50 mM Tris–HCl (pH 7.5), at 20 °C (Chelomin and Zhukova, 1981) for different time intervals (within range from 5 to 90 min). The reaction was terminated by acidification to pH 5.0 with 5% acetic acid and followed by centrifugation at $10\,000 \times g$ for 15 min. To remove unbound TNBS, the membrane pellet was washed twice in 150 mM NaCl, 50 mM Tris–HCl (pH 7.5) buffer.

Lipids were extracted by the technique of Bligh and Dyer (1959). Two-dimensional micro-thin-layer chromatography, identification of trinitrophenyl (TNP)-derivatives of aminophospholipids (phosphatidylethanolamine—PE; phosphatidylserine—PS) and its quantitative determination were carried out by methods described earlier (Chelomin and Zhukova, 1981). Content of TNP-derivatives of the aminophospholipids was measured as a function of treatment time (within range from 5 to 90 min).

2.4. Miscellaneous methods

The content of sulfhydryl groups (SH-group) in the membrane fraction was measured by its reaction with Ellman's reagent (Haest et al., 1978). The amount of total synaptosomal protein was determined by bromphenol dye (Greenberg and Gaddock, 1982) with bovine serum albumin (BSA) as a standard. Induction of lipid peroxidation (LP) of the synaptosomal membrane (about 100 μg of total membrane protein) was initiated by ascorbic acid and Fe^{2+} (as $FeSO_4$) with a final concentration 50 and 10 μM, respectively (Kreps et al., 1987). The reaction lasted 90 min at a temperature of 25 °C, and then 2,[6]-di-*tert*-butyl-*p*-crezol (as well known as a ionol) at a final concentration of 5 μM was added to terminate it. The level of lipid peroxidation was estimated by the amount of substances that react with 2-thiobarbituric acid (TBA) and quantified in terms of malonaldehyde (MDA) equivalents (Burk et al., 1980).

2.5. Statistics

The observed kinetics of productions of the TNP-derivatives of aminophospholipids were fitted to Michaelis–Menten equation, because it has lowest minimal absolute sum of squares (as the fitting criterion) in comparing with others models. Therefore, we have calculated saturation level (L_{sat}) as well as maximal rate of reaction (V_{max}) for the mentioned equation by means of nonlinear regression analysis (GraphPad Pism V 2.0 software). Kinetic measurements were repeated four times ($n = 4$) on the synaptosomes obtained from each single capture. Saturation levels are represented as mean and standard error (mean ± S.E.). Significant differences of L_{sat} were evaluated by repeated measures one-way ANOVA (GraphPad Instat V 3.01 software) with Tukey posttest. Differences are significant at $P < 0.001$.

Measurements of SH-group content and lipid peroxidation level were repeated ten times ($n = 10$) on the synaptosomes obtained from several captures. Data are represented as mean and standard deviation (mean ± S.D.). Significant differences were evaluated by one-way ANOVA (GraphPad Instat V 3.01 software) with Tukey posttest. Differences are significant at $P < 0.05$.

3. Results and discussion

Trinitrobenzenesulfonate has been a widely used reagent for assessing aminophospholipid distribution in membranes of different origin and in membranes of various groups of animals (Bretscher, 1972; Estemadi, 1980). Marine invertebrates and fish are no an exception (Bretscher, 1972; Chelomin and Zhukova, 1981; Chelomin and Sizov, 1986). Trinitrobenzenesulfonate is regarded as a non-penetrating reagent because of its charged sulfonic group. Using this reagent, the transbilayer asymmetry of aminophospholipids in different membranes was revealed. Specifically, some research groups (Gordesky and Marinetti, 1973; Estemadi, 1980; Chelomin and Zhukova, 1981; Chelomin and Sizov, 1986) observed that 75–80% of the total phosphatidylethanolamines (PE) and about all of phosphatidylserines (PS) were localized into the inner monolayer of plasma membrane.

The kinetics of the reaction between TNBS and aminogroups of synaptosomal PE and PS are shown on Fig. 1(a, b). From the kinetic analysis of the curves (Table 1) we can conclude that $23.2 \pm 1.1\%$ of total PE and $2.2 \pm 0.03\%$ of total PS were localized in the outer membrane monolayer. Thus, distribution of aminophospholipids into the synaptosomes of walleye pollock *T. chalcogramma* followed the common trend of aminophospholipid asymmetry in membrane.

Preliminary treatment of synaptosomes with copper ions (Cu^{2+}, 100 μM, 90 min at 20 °C) changed accessibility of aminophospholipids for TNBS. The amount of TNP-derivatives of PE and PS increased 2.2 and 14 times, respectively (Fig. 1a and b; Table 1). This phenomenon suggests that the general asymmetry of the membrane was perturbed as a result of this treatment.

From the above data the question arises: what is the mechanism of this effect of copper ions on the membrane?

Literature data show that membrane proteins are quite assailable for some heavy metals ions because they contain reduced sulfhydryl groups (SH-groups) which have a very high affinity for heavy metal ions, especially for Hg^{2+}, Cd^{2+} and Cu^{2+} (Christie and Costa, 1984).

In particularly, copper ions easily oxidize SH-groups of cysteine from membrane proteins by the following mechanism:

$$Cu^{2+} + 2SH\text{–}R \rightarrow R\text{–}SR + Cu^{+} + 2H^{+}$$

(Freedman et al., 1989) (1)

Intra- and intermolecular ‘disulfide bridges’ are formed between membrane proteins as a result of this reaction. In case of mammalian erythrocytes such a process leads to deformation and hemolysis of the erythrocytes (Salhany et al., 1978; Asano and Hokari, 1985; Ito and Kon, 1987). Similar results were observed with erythrocyte from mollusk *Scapharca broughtoni*, which had been treated with copper ions (Chelomin and Busev, 1986). Appearance of polymerized proteins was observed in the membrane fractions as a result of such treatment.

Our results show that the concentration of sulfhydryl groups significantly decreased (3.6 times) in Cu^{2+}-treated synaptosomes (Table 2). We assume that the oxidation of SH-groups of membrane proteins is an initial cause of reorientation of aminophospholipids in the membrane. Our assumption is based on two main reasons. First, absolute (functional) asymmetry of membrane protein dictates the relative (quantitative) asymmetry of membrane phospholipids (Rothman and Lenard, 1977). Second, transmembrane distributions of aminophospholipids (PE, PS in particular) are retained with participation of Ca^{2+}–ATPases (Zachowski et al., 1985; Bitbol et al., 1987; Middelkoop et al., 1988). Therefore, this information gives us the base to assume that disturbance of the membrane protein framework appears to be the reason of reorientation of aminophospholipids into the membrane. The same conclusion was drawn by Haest and

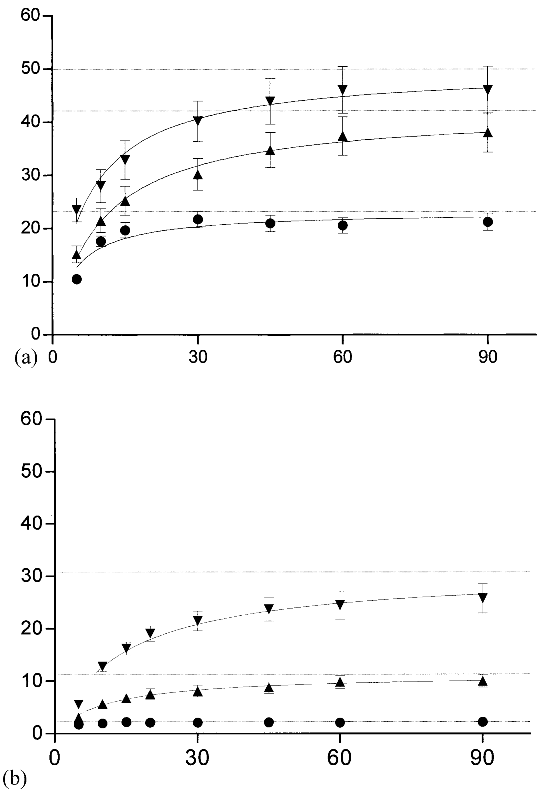

Fig. 1. Kinetics of TNBS reaction with membrane's phosphatidylethanolamine (PE, a) and phosphatidylserine (PS, b) in control conditions (●), after treatment with Cu^{2+} ions (100 μM, 90 min at 20 °C) (▼) or induction of Fe–ascorbate lipid peroxidation (LP, conditions see in text) (▲). Values are mean ± S.D. ($n = 4$). Abscissa—time in minutes. a ordinate—% of TNP-derivates of PE from total content of the lipid; b ordinate—% of TNP-derivates of PS from total content of the lipid; horizontal dotted lines—saturation level (L_{sat}) of TNP-derivates of correspondent lipids (see Table 1).

Table 1
Saturation level (L_{sat}, value ± S.E., $n = 4$) of TNP-derivates of phosphatidylethanolamine (PE) and phosphatidylserine (PS) as a percentage of total content of the lipid in fraction of synaptosomal membranes from brain of walley pollock *T. chalcogramma* after treatment with Cu^{2+} ions (100 μM, 90 min at 20 °C) or induction of Fe–ascorbate lipid peroxidation (LP, conditions see in text)

	L_{sat} of TNP–PE (% of total PE)	L_{sat} of TNP–PS (% of total PS)
Control	23.2 ± 1.1	2.2 ± 0.03
Cu^{2+}	50.0 ± 1.3	30.8 ± 1.4
LP	42.2 ± 1.0	11.4 ± 0.3

$P < 0.001$.

Table 2
Contents (mean ± S.D., $n = 10$) of reduced sulfhydryl groups (SH-groups) and malone dialdehyde (MDA) in fraction of synaptosomal membranes from brain of walley pollock *T. chalcogramma* after treatment with Cu^{2+} ions (100 μM, 90 min at 20 °C) or induction of Fe–ascorbate lipid peroxidation (LP, conditions see in text)

	Concetration of SH-groups (μmol SH-groups/mg protein)	Concentration of MDA (nmol MDA/mg protein)
Control	58.6 ± 1.7	1.21 ± 0.12
Cu^{2+}	16.3 ± 2.6***	2.34 ± 0.47*
LP	27.7 ± 2.4***	8.93 ± 1.31***

* $P < 0.05$.
*** $P < 0.001$.

Deuticke (1976), who found that SH-group oxidizing agents (hydroquinone and sodium tetrathionate) are able to facilitate the reorientation of phospholipids into membranes.

However, there is another possibility for the destructive processes to act. According to Eq. (1) copper ion accepts one electron from SH-group reducing itself from Cu^{2+} to Cu^{+}, which (Cu^{+}) are able to return back into Cu^{2+} state by means of giving an electron to molecular oxygen and activating it and thus creating a superoxide radical ($O_2^{\bullet -}$, Eq. (2))

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{\bullet -}$$

(Freedman et al., 1989) (2)

Reactions 1 and 2 take place very close to membrane surface, thus the superoxide radical is able to permeate into the membrane and induce a lipid peroxidation (LP) with free radical mechanism.

$$O_2^{\bullet -} + \mathrm{RH\ (lipid)} \rightarrow \mathrm{ROOH}$$

(Freedman et al., 1989) (3)

We have observed that in the Cu-treated synaptosomes, the concentration of the MDA increased almost twice, compared to control (Table 2).

Different products of lipid peroxidation (such as MDA, peroxide radicals, hydroperoxide of fatty acids) are able to elicit changes in physical-chemical and biochemical properties of membranes (such as fluidity, electrical resistance, interlayer exchange with phospholipids, inhibition of membrane associated and bound enzymes, and disruption of barrier function of membrane) (Panasenko et al., 1985; Richter, 1987; Zakhartsev et al., 2000). From this we expect the same effect of LP on transbilayer distribution of phospholipids.

We have induced non-enzyme LP (Fe–ascorbate) in the synaptosomal fraction of the membrane to check this hypothesis. The levels of MDA increased almost 7.5 times as a result of this treatment (Table 2). Further reaction with TNBS reveals that the amount of PE in the outer monolayer increased 1.8 times and PS 5.2 times, respectively, (Fig. 1a and b; Table 1). These results confirm our suspicion that LP can affect transbilayer distribution of aminophospholipids.

There is no doubt that both of these processes take place in the membrane and each may be a primary cause of aminophospholipids reorganization in the case of a Cu intoxication. However, the accessibility of the SH-groups and the ratio of anti- and prooxidative properties will determine the priority of each particular process.

Synaptosomal plasmatic membranes bear the common features that are inherent to both surface and intracellular biological membranes. We assume that the mentioned functional damages of the lipid matrix would take place in the intracellular membranes as a result of copper accumulation. Moreover, reduced glutathione (GSH) can play a role as a copper reducing agent (Freedman et al., 1989).

Copper-induced reorganization of membrane aminophospholipids is not an unusual occurrence. Externalization of membrane PS was a consistent feature in *Plasmodium knowlesi* infected erythrocytes of monkey and human (Gupta, 1988). Apoptosis is triggered by specific surface changes, the most familiar of which is the externalization of PS in the plasma membrane (Fadok et al., 1992).

Copper is a potentially toxic element for any biological system. There are numbers of biochemical shifts that were provoked by excess of copper, but we cannot fully evaluate the degree of noted effects in the whole pathological process.

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