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Increased Copper-Mediated Oxidation of Membrane Phosphatidylethanolamine in Autism

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Abstract: We have previously reported that levels of phosphatidylethanolamine (PE) in the erythrocyte membrane and of ceruloplasmin, a copper-transport antioxidant protein, in the serum are lower in children with autism than in control subjects. In the present study, we report that (a) copper oxidizes and reduces the levels of membrane PE and (b) copper-mediated oxidation of PE is higher in lymphoblasts from autistic subjects than from control subjects. The effect of copper was examined on the oxidation of liposomes composed of brain lipids from mice and also on the lymphoblasts from autism and control subjects. Among the various metal cations (copper, iron, calcium, cadmium and zinc), only copper was found to oxidize and decrease the levels of PE. The metal cations did not affect the levels of other phospholipids. The action of copper on PE oxidation was time-dependent and concentration-dependent. No difference was observed between copper-mediated oxidation of diacyl-PE and alkenyl-PE (plasmalogen), suggesting that plasmalogenic and non-plasmalogenic PE are equally oxidized by copper. Together, these studies suggest that ceruloplasmin and copper may contribute to oxidative stress and to reduced levels of membrane PE in autism.

Key words: Autism, copper, lymphoblast, membrane, metals, oxidation, phospholipids

INTRODUCTION

Autism is a severe neurodevelopmental disorder with a poorly understood etiology. Recent evidence from our laboratory and others suggests that oxidative stress may be an important component in autism[1-8]. We have reported that lipid peroxidation is greater in the plasma of children with autism than in developmentally normal siblings[5]. Increased urinary excretion of 8-hydroxy-2-deoxyguanosine (oxidative product of DNA) and 8-isoprostane-F2α (oxidative product of fatty acids) has been reported in children with autism[7]. An increased vulnerability to oxidative stress and decreased capacity for methylation has been reported by James *et al*[8]. Zoroglu *et al.* reported altered activities of free radical scavenging enzymes in erythrocytes from autism[3].

Copper (Cu), a trace metal, is an essential element for living cells. It plays an important role in redox reactions because of its easy conversion from Cu^{+} to Cu^{++}. Copper is transported mainly by ceruloplasmin, a copper-binding antioxidant protein that is synthesized in several tissues, including brain[9,10]. Ceruloplasmin inhibits the peroxidation of membrane lipids catalyzed by metal ions, such as iron and copper[11]. It also acts as ferroxidase and superoxide dismutase and it protects polyunsaturated fatty acids in red blood cell membranes from active oxygen radicals[10]. Copper levels are low in Menke's kinky hair syndrome[12], malnutrition[13] and malabsorption[14]. Its elevated levels are associated with infections[15], inflammation[16], trauma[17], Wilson's disease[18], excessive dietary intake[19] and systemic lupus erythematosus[20]. The majority of copper is bound to ceruloplasmin and the remainder is bound to albumin, metallothionein and other proteins. This bound copper is not redox-active. However, it is toxic in its unbound form. We have reported reduced levels of serum ceruloplasmin in children with autism, suggesting that copper metabolism may be altered in autism[5]. It was of particular interest to observe that levels of ceruloplasmin were reduced more significantly in children with autism who had lost previously acquired language skills[5]. Phosphatidylethanolamine (PE) levels were also decreased in the erythrocyte membranes from autism than from control subjects[6].

In this study, we examined the effect of copper on the oxidation of brain lipids in the liposomes and on the lymphoblasts of autism and control subjects. We observed that copper predominantly oxidizes PE in the membrane and copper-mediated PE oxidation is higher in autistic lymphoblasts as compared to control lymphoblasts.

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MATERIALS AND METHODS

Materials: Thin layer chromatography (TLC) silica gel 60 plates were purchased from VWR. All chemicals were from Sigma Chemical Co.

Extraction of brain lipids: Lipids from brains of mice were extracted in chloroform: methanol; 2:1 (V/V). Phospholipids were measured by digesting the lipids in 70% perchloric acid and estimating the amount of inorganic phosphorus. The lipid phosphorus was multiplied by a factor of 25 to obtain the phospholipid content in the sample. The stock solution of brain phospholipids was prepared in chloroform (5 mg mL^{-1}) and stored at –20 ^{0}C.

Preparation of liposomes: Five hundred μg of the brain phospholipids above were dried under nitrogen and hydrated in 500 μL phosphate-buffered saline, pH 7.4, for 30 min. at room temperature, followed by probe sonication on ice for 20 min. under a stream of nitrogen.

Lymphoblasts: Lymphoblasts from autistic and control subjects were obtained from the Autism Genetic Resource Exchange (AGRE). Cells were grown in RPMI 1640 containing 17% fetal bovine serum, 100 mg mL^{-1} penicillin and 100 mg mL^{-1} streptomycin. The cells were fed every 4th day.

Effect of metal ions on the oxidation of phospholipids in liposomal membrane: Sixty μL liposomes (prepared from mouse brain lipids as described above) and 10 μM metal ions ($CuCl_2$, $FeCl_3$, $ZnCl_2$, $CdCl_2$ and $CaCl_2$) in a total volume of 100 μL were incubated at 37^{0}C. After 8 hr, lipids were extracted using chloroform/methanol (2:1, V/V). Lipids were dried under nitrogen and solubilized in 50 μL chloroform, followed by the addition of another 50 μL chloroform (a total of 100 μL). These samples and lipid standards of sphingomyelin (SPG), phosphatidylserine (PS), phosphatidylcholine (PC) and PE in chloroform were spotted on a TLC plate. The plate was developed in a chloroform/methanol/water (65/25/4; V/V) solvent system. The lipid spots were visualized by placing a TLC plate in iodine chamber. The spots in the experimental samples corresponding to PE, PC, PS and SPG standards were scraped from the plate and the amount of phospholipid phosphorus was estimated in these samples as described previously[21].

Effect of different concentrations of copper on oxidation of PE in liposomes of brain lipids: 60 μL liposomes were incubated at 37^{0}C with various concentrations of copper (2 – 10 μM). After 8 hr, the lipids were extracted and separated on TLC as described above. The lipids were visualized by iodine vapors. The PE spot was scraped and phospholipid phosphorus was measured.

Effect of incubation time on copper-mediated oxidation of brain PE in liposomes: Ten μM copper and 60 μL liposomes of brain lipids were incubated in a total volume of 100 μL at 37^{0}C for different time periods i.e. 30 min and 1, 2, 4, 6 and 8 hr. The lipids were extracted and separated. The phospholipid phosphorus was measured in PE spot.

Effect of copper on the oxidation of PE-plasmalogens: Because HCl vapors break the alkenyl group of phospholipids while having no effect on diacyl PE, PE-plasmalogens get hydrolyzed under HCl vapors. 60 μL liposomes of brain lipids were incubated with 10 μM Cu^{2+} at 37^{0}C. After 8 hr, the lipids were extracted and separated on TLC plate as described above. The TLC plate was dried and exposed to HCl vapors by keeping the plate face down. After 30 min., the TLC plate was taken out and HCl vapors were removed by blowing hot air. Finally, the plate was developed in second dimension using a solvent system of chloroform/methanol/water (65/25/4; V/V). Two spots of PE were observed, the lower spot corresponding to PE plasmalogen due to the cleavage of alkenyl group from PE and the top spot corresponding to diacyl PE. Phosphorus contents in both spots were measured as described previously[21].

Effect of copper on oxidation of PE in lymphoblasts from autistic and control subjects: Lymphoblasts from two autistic and two control subjects were washed in serum-free medium. 8 x10^6 cells were incubated overnight with 200 μM and 400 μM $CuCl_2$ in CO_2 incubator. Lipids were extracted and phospholipids were separated on TLC plate. The phospholipid phosphorus was then measured in the PE spot of the samples.

RESULTS

Metal ion-mediated oxidation of lipids: Figure 1 shows the oxidation of lipids in the presence of 10 μM of different metal ions (Cu^{2+}, Fe^{3+}, Zn^{2+}, Ca^{2+} and Cd^{2+}). Among the metal ions tested, oxidation of

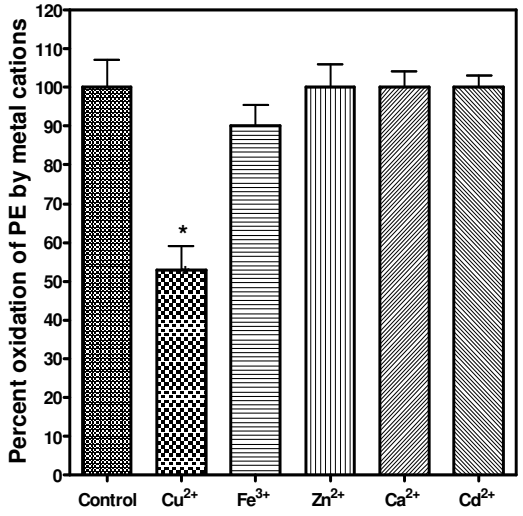

Fig. 1: Effect of various metal cations on the oxidation of PE. 60 µL liposomes containing 60 µg phospholipids were incubated with 10 µM metal ions ($CuCl_2$, $FeCl_3$, $ZnCl_2$, $CdCl_2$ and $CaCl_2$) at 37^0C. In the control sample, metal ion was not added. After 8 hr, the lipids were extracted and separated on TLC and phospholipid phosphorus was measured. Percent effect of metal cations was calculated by using phosphorus content in the control sample as 100. Values are mean ± S.E. of three experiments. * denotes $p < 0.01$ as compared to control sample

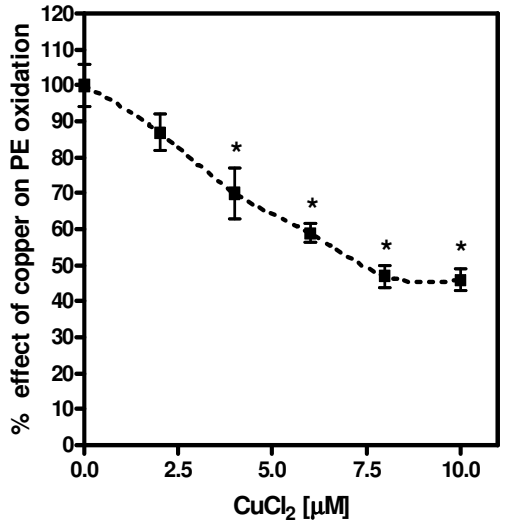

Fig. 2: Concentration-dependent copper-mediated oxidation of PE. 60 µL liposomes (60 µg of brain phospholipids) were incubated with the indicated concentrations of Cu^{2+}. After 8 hr, lipids were extracted and separated and phosphorus was measured in the PE spot. Percent effect of Cu^{2+} was calculated by using phosphorus content in the control sample as 100. Values are mean ± S.E. of three experiments. * denotes $p < 0.01$ as compared to control sample

phospholipids was observed only with Cu^2. Among the phospholipids, only PE was oxidized significantly (50%) by Cu^{2+}, as indicated by the reduced contents of PE in the presence of Cu^{2+} (Fig. 1). There was no effect of Cu^{2+} or other metal cations on other phospholipids, i.e. PC, SPG and PS (data not shown).

Concentration-dependent oxidation of brain PE by copper: The effect of different concentrations of Cu^{2+} on oxidation of membrane PE is shown in Fig. 2. Incubation of liposomes composed of brain lipids from mice with various concentrations of copper (2 µM –10 µM) resulted in oxidation of PE in a concentration-dependent manner from 2 µM – 8 µM Cu^{2+} that was followed by a plateau. Significant oxidation of PE ($p<0.01$) was observed at the copper concentration of 4 µM – 10 µM.

Time-dependent oxidation of PE by copper: When liposomes of mouse brain lipids were incubated with 10 µM copper for different time periods, copper increased the oxidation of PE in a time-dependent manner (Fig. 3). There was an initial 1 hr lag phase. An increase in Cu^{2+}-mediated PE oxidation was observed from 1 hr to 4 hr that was followed by plateaus at 6 hr and 8 hr. The

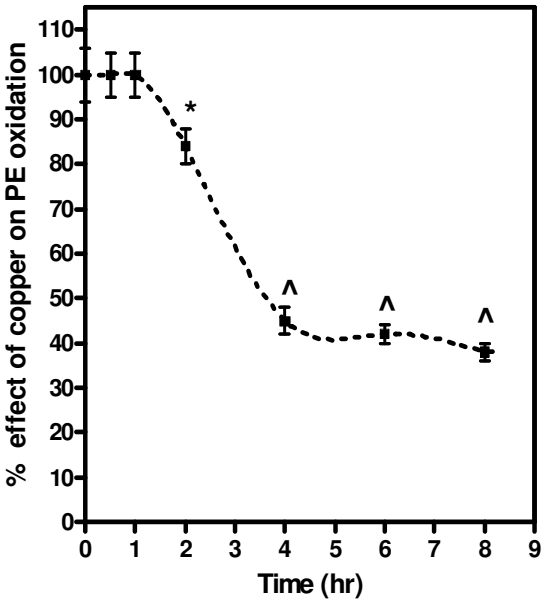

Fig. 3: Time-dependent copper-mediated oxidation of PE. 60 µL liposomes (60 µg brain phospholipids) were incubated with 10 µM Cu^{2+}. At the indicated time periods, lipids were extracted and separated on TLC plate and phosphorus in the PE spot was measured. Percent effect was calculated by using phosphorus content in the control as 100. Values are mean ± S.E. of three experiments. * denotes $p < 0.05$ and ^ denotes $p < 0.01$ as compared to control sample

effect of Cu^{2+} on PE oxidation was significant from 2 hr to 8 hr.

Oxidation of alkenyl-PE (plasmalogen) and diacyl-PE by copper: Brain PE exists in alkenyl-PE form, i.e. PE-plasmalogen and diacyl-PE form. Copper was observed to significantly oxidize ($p<0.01$) both diacyl PE and alkenyl PE to a similar extent (Fig. 4).

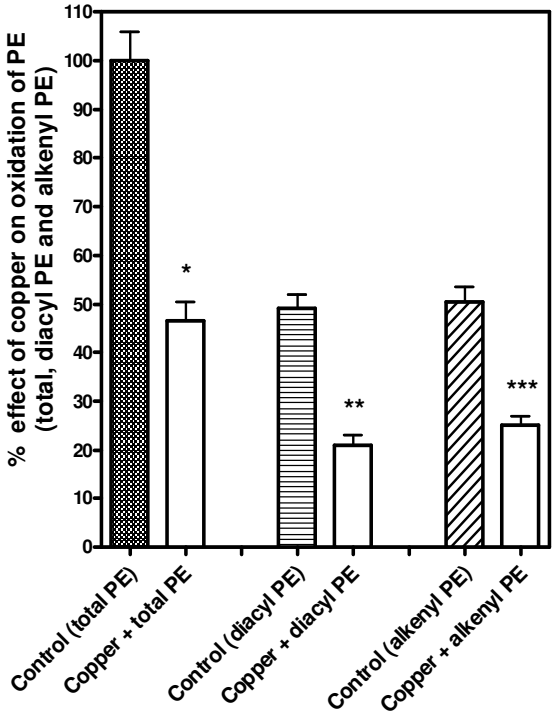

Fig. 4: Copper-mediated oxidation of PE-plasmalogen. 60 µL liposomes (60 µg of brain phospholipids) were treated with 10 µM copper at 37^0C for 8 hr. The plasmalogenic PE and diacyl PE were separated and the phosphorus content was measured. Percent effect was calculated by using phosphorus content in the control as 100. Values are mean ± S.E. of three experiments. * denotes $p < 0.01$ as compared to control (total PE); ** denotes $p < 0.01$ as compared to control (diacyl PE); *** denotes $p < 0.01$ as compared to control (alkenyl PE)

Increased copper-mediated oxidation of PE in autistic lymphoblasts: Cu^{2+} could significantly induce oxidation of PE in lymphoblast membrane as evidenced by reduced ($p<0.01$) PE levels in Cu^{2+}- treated lymphoblasts from autism subjects and control subjects than in untreated samples (Fig. 5). The oxidation of PE by copper (200 µM and 400 µM) in lymphoblasts from autistic subjects was significantly higher ($p<0.05$) as compared to lymphoblasts from control subjects.

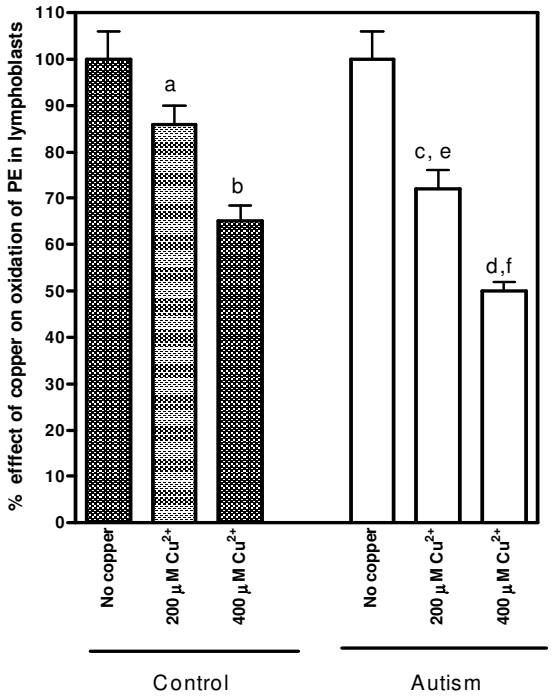

Fig. 5: Effect of copper on the PE of lymphoblasts from autism and control subjects. 8 x 10^6 lymphoblasts from autistic and control subjects were incubated with the indicated concentrations of copper chloride. After 14 hr, the cells were washed with Tris-buffered saline and lipids were extracted and separated on TLC. The phospholipid phophorus was measured. Percent effect was calculated by using phosphorus content in the control as 100. Values are mean ± S.E. of two experiments done with two control lymphoblasts and two autistic lymphoblasts, [a] denotes $p < 0.05$ as compared to control (no copper); [b] denotes $p < 0.01$ as compared to control (no copper); [c] denotes $p < 0.01$ as compared to autism (no copper); [d] denotes $p < 0.01$ as compared to autism (no copper); [e] denotes $p < 0.05$ as compared to control lymphoblasts treated with 200 µM copper; and [f] denotes $p < 0.05$ as compared to control lymphoblasts treated with 400 µM copper

DISCUSSION

Increasing evidence suggests oxidative stress in autism[1-5,7,8,22-25]. Unsaturated fatty acids in phospholipids are the prime target of free radical–mediated oxidation. We reported previously that levels of ceruloplasmin in the serum and PE in the erythrocyte membrane are lower in children with autism than in their unaffected siblings[5]. Ceruloplasmin is a main copper-transporting protein. Decreased ceruloplasmin levels suggest that there may be an imbalance in the copper metabolism in autism. Several reports indicate an important role of copper ions in the oxidation of

lipids[26-33]. Therefore, we examined the effect of copper and other metal cations on brain phospholipids.

Among the metal cations, copper had a pronounced effect on oxidation of lipids and it oxidized primarily PE in the liposomal membrane composed of the mouse brain lipids. Because brain lipids contain PE-plasmalogens, we also examined whether copper affects the oxidation of PE-plasmalogens. Indeed, copper was found to oxidize both plasmalogenic and non-plasmalogenic PE in the liposomes composed of brain lipids. We did not observe oxidation of PC by Cu^{2+}, as others had reported [32,33] in liposomes composed of only PC. It has been reported that plasmalogens of PE can delay or prevent the oxidation of lipids[26,27,33]. Because brain lipids contain PE-plasmalogens, it is possible that these plasmalogens protected PC from copper-mediated oxidation. Our results suggest that although PE-plasmalogens protected against oxidation of other phospholipids, it may have delayed but could not protect against oxidation of diacyl-PE, i.e. non-plasmalogenic PE. The action of copper on oxidation of PE was in a time- and concentration-dependent manner. It was a slow reaction requiring several hours of incubation. Reiss et al.[26] and Hahnel et al.[33] observed a similar effect of copper on oxidation of lipids. We observed that copper-mediated oxidation had a lag period of 1 hr, followed by a significant increase till 4 hr. It seems that PE was rapidly oxidized after the reaction initiated.

A question arises: why of all the phospholipids, is PE prone to oxidation by copper? This may be because of the primary amino group in PE, i.e. PE-NH_2. Amines react with copper ions in two separate stages: in the first step, the Bronsted-Lowry theory (that a base is a hydrogen ion acceptor) applies; and in the next step, the Lewis theory (that a base is an electron pair donor) applies. Copper chloride solution contains the blue hexaaquacopper (II) ion, i.e. $[Cu(H_2O)_6]^2$. In the first step of the copper reaction with PE, the amine will pull off hydrogen ions off two attached water molecules:

$$[Cu(H_2O)_6]^{2+} + 2\ PE\text{-}NH_2 \rightarrow [Cu(H_2O)_4(OH)_2] + 2PE\text{-}NH_3^+$$

With more PE-NH_2, the amine will replace four of the H_2O molecules around copper:

$$[Cu(H_2O)_6]^{2+} + 4\ PE\text{-}NH_2 \rightarrow [Cu(PE\text{-}NH_2)_4(H_2O)_2]^{2+} + 4H_2O$$

As the PE-NH_2 gets bigger and bulkier, the formula of the final product may change because four large amine molecules and two H_2O molecules will not be able to fit around the copper atom.

In addition to the interaction of copper with PE-NH_2, copper is also involved in catalyzing free radical-generation, a prerequisite for oxidation of lipids. Copper catalyzes the formation of free radicals as follows:

$$Cu^+ + ROOH \rightarrow RO^* + {}^-OH + Cu^{++}$$

$$Cu^{++} + ROOH \rightarrow ROO^* + H^+ + Cu^+$$

where R is an alkyl group and molecules with * are free radicals generated during reactions, which eventually will oxidize phospholipids.

We also examined the effect of copper on the oxidation of PE in lymphoblasts from autism and control subjects. While copper oxidized PE in both autistic and control lymphoblasts, the oxidation of PE was significantly higher in lymphoblasts from autism subjects than from control subjects. These results support our previous observation of lower levels of PE in the erythrocyte membrane from autism subjects than in their developmentally normal siblings[6]. We also reported that levels of PS are greater in the erythrocyte membranes of children with autism than in unaffected siblings[6]. PE is a zwitterionic phospholipid, whereas PS is acidic. Therefore, PE may be more susceptible to copper-mediated oxidation because of its neutral charges at physiological pH.

Together, these studies suggest that decreased levels of ceruloplasmin and abnormal copper metabolism in autism may be contributing factors inducing oxidative stress and membrane abnormalities in autism subjects.

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