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Aluminum Decreases the Glutathione Regeneration by the Inhibition of NADP-Isocitrate Dehydrogenase in Mitochondria

Keiko Murakami and Masataka Yoshino*

Department of Biochemistry, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan

Abstract Effect of aluminum on the NADPH supply and glutathione regeneration in mitochondria was analyzed. Reduced glutathione acted as a principal scavenger of reactive oxygen species in mitochondria. Aluminum inhibited the regeneration of glutathione from the oxidized form, and the effect was due to the inhibition of NADP-isocitrate dehydrogenase the only enzyme supplying NADPH in mitochondria. In cytosol, aluminum inhibited the glutathione regeneration dependent on NADPH supply by malic enzyme and NADP-isocitrate dehydrogenase, but did not affect the glucose 6-phosphate dehydrogenase dependent glutathione formation. Aluminum can cause oxidative damage on cellular biological processes by inhibiting glutathione regeneration through the inhibition of NADPH supply in mitochondria, but only a little inhibitory effect on the glutathione generation in cytosol. J. Cell. Biochem. 93: 1267–1271, 2004. © 2004 Wiley-Liss, Inc.

Key words: aluminum; glutathione; NADP-isocitrate dehydrogenase; malic enzyme

Aluminum the most abundant element in the earth crust has been implicated as a potential, contributory factor in the etiology and pathology of some neurodegenerative diseases [Ganrot, 1986]. Combined experimental, clinical, and epidemiological evidences suggest that aluminum may be a neurotoxic in humans [Meiri et al., 1993; Strong, 1994], and increased aluminum levels in the brain are recognized as a causative agent in human encephalopathy [McLachlan et al., 1991; Bolla et al., 1992; Candy et al., 1992; Good et al., 1992]. One of the mechanisms underlying aluminum-mediated cellular toxicity is considered to be the inhibition of the energy metabolism [Womack and Colowick, 1979; Yoshino et al., 1990] and the prooxidant action causing lipid peroxidation [Fraga et al., 1990]. Recent studies showed the prooxidant activity of aluminum to be due to the formation of an aluminum superoxide semiquinone radical ion [Exley, 2004]. Previously, we presented a potent inhibitory effect of aluminum on NADP-isocitrate dehydrogenase (EC 1.1.1.42) from yeast and porcine heart [Yoshino and Murakami, 1992; Yoshino et al., 1992]. NADP-isocitrate dehydrogenase acts as an enzyme of the citric acid cycle and further as an antioxidant enzyme that produces reduced NADP for the regeneration of reduced glutathione. Inhibition of the NADPH-generating enzyme(s) by aluminum may cause an oxidative injury to cells. In this paper, we report that aluminum can inhibit the regeneration of reduced glutathione responsible for scavenging reactive oxygen species: the inhibition of the glutathione regeneration was due to the decreased supply of NADPH by the inhibition of NADP-isocitrate dehydrogenase in mitochondria.

MATERIALS AND METHODS

Materials

The sources of materials in this work were as follows: threo-DS-isocitrate from Sigma-Aldrich (Tokyo, Japan), heart mitochondrial NADP-isocitrate dehydrognease, chicken liver malic enzyme (EC 1.1.1.38), NADP, and reduced

*Correspondence to: Masataka Yoshino, Department of Biochemistry, Aichi Medical University School of Medicine, Nagakute, Aichi 480-1195, Japan.
E-mail: yoshino@aichi-med-u.ac.jp

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glutathione from Roche Diagnostics (Tokyo, Japan), 5,5′-dithiobis-(2-nitro-benzoic acid) from Seikagaku Corporation (Tokyo, Japan). Other chemicals were obtained from commercial sources.

Determination of Enzyme Activities

NADP-isocitrate dehydrogenase activities were determined by following the change in absorbance at 340 nm as described previously [Bolla et al., 1992]. Malic enzyme activity was determined according to the method described by Outlaw and Springer [Outlaw and Springer, 1983]. The assay mixture of 1.0 ml contained 100 mM Tris-HCl buffer (pH 7.1), 0.1 mM NADP, 1 mM malate, 1 mM dthiothreitol, 8 mM $MgCl_2$, the enzyme, and various concentrations of aluminum chloride. The reaction was initiated by the addition of the enzyme, and incubated at 37°C. The increase in the absorbance at 340 nm was recorded.

Formation of Reduced Glutathione in Mitochondria and Cytosol

Mitochondrial and cytosolic fractions were isolated from the liver of adult male Sprague–Dawley rats by standard fractionation method [Matlib et al., 1979]. Formation of reduced glutathione was monitored by the reaction of glutathione with 5,5′-dithiobis-(2-nitrobenzoic acid) as the increase in the absorbance at 412 nm. Reaction mixture of 1 ml contained 0.5 mM oxidized glutathione, 0.1 mM NADP, 2 mM $MgCl_2$, 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid), mitochondrial, or cytosolic fractions of 0.5–1 mg/ml, 40 mM Tris-HCl buffer (pH 7.1), and the substrates for NADPH-generating system including 0.1 mM threo-DS-isocitrate, 1 mM glucose 6-phosphate, or 5 mM malic acid in the absence and presence of aluminum chloride.

RESULTS

Essential role of glutathione regeneration in scavenging hydrogen peroxide was examined with isolated mitochondria. Mitochondrial fraction was incubated with reduced glutathione and NADPH, and addition of hydrogen peroxide depleted NADPH markedly (Fig. 1). This indicates that glutathione peroxidase (EC 1.11.1.9) scavenging hydrogen peroxide with glutathione produced oxidized glutathione, which was regenerated to reduced glutathione by glutathione reductase (EC 1.6.4.2) with NADPH. Depletion of NADPH indicates the consumption

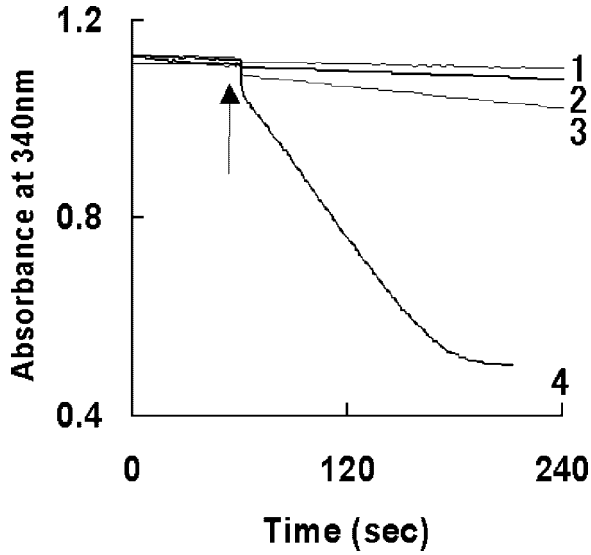

Fig. 1. NADPH-dependent glutathione peroxidase activity scavenging hydrogen peroxide in mitochondria from rat liver. Reaction mixture of 1 ml containing 0.09 mM hydrogen peroxide, 2 mM reduced glutathione, 0.2 mM NADPH, 0.2 U/ml glutathione reductase, 0.5 mM sodium azide, and 50 mM potassium phosphate buffer (pH 7.0) was incubated with mitochondrial fraction at 37°C. Reaction of glutathione peroxidase scavenging hydrogen peroxide with reduced glutathione was followed by formation of oxidized glutathione, which was regenerated to reduced glutathione by glutathione reductase with NADPH. Decrease in glutathione concentration corresponds to the decrease in the absorbance at 340 nm. Curve 1: Mitochondria and glutathione omitted; (Curve 2) glutathione omitted (mitochondria added); (Curve 3) mitochondria omitted (2 mM glutathione added); (Curve 4) mitochondria with glutathione added (complete system). Arrow indicates the addition of hydrogen peroxide.

of glutathione by glutathione peroxidase in mitochondria, and the supply of NADPH is suggested to be a key factor for scavenging hydrogen peroxide.

We further examined the source of NADPH for the reduction of oxidized glutathione in mitochondria. Oxidized glutathione was incubated with NADP in mitochondrial fraction, and addition of isocitrate effectively generated reduced glutathione; however, little of no formation of glutathione was observed by addition of glucose 6-phosaphate or malate (Fig. 2). Addition of glucose 6-phosphate and glucose 6-phosphate dehydrogenase formed reduced glutathione markedly (Fig. 2). These results indicate that reduced equivalents for regeneration of glutathione resulted from NADP-isocitrate dehydrogenase in mitochondria.

Effect of aluminum on the formation of reduced glutathione was examined with mitochondria from rat liver. Reduction of GSSG to

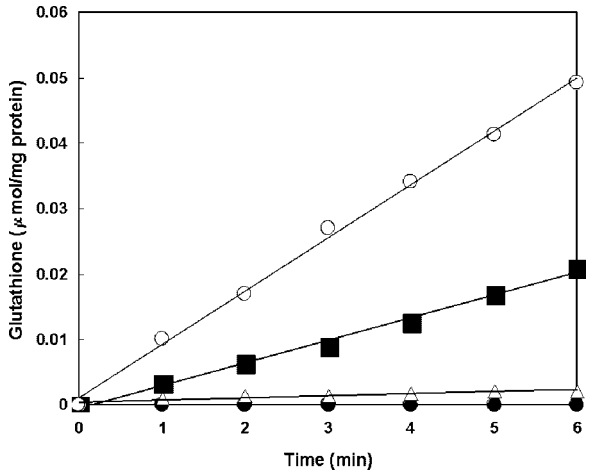

Fig. 2. Reduction of oxidized glutathione dependent on different NADPH-generating system in mitochondrial fractions. Reduced glutathione was determined by the reaction of glutathione with 5,5′-dithiobis-(2-nitrobenzoid acid) as the increase in the absorbance at 412 nm. Reaction mixture of 1 ml contained 0.1 mM NADP, 1 mM oxidized glutathione, 2 mM $MgCl_2$, 40 mM Tris-HCl buffer (pH 7.1), 0.2 mM 5,5′-dithiobis-(2-nitrobenzoid acid), mitochondrial fractions, and various NADPH-generating systems as follows: ■, 0.1 mM threo-DS-isocitrate; △, 1 mM glucose 6-phosphate; ○, 1 mM glucose 6-phosphate plus 5 U/ml glucose 6-phosphate dehydrogenase; ●, 5 mM malate.

reduced glutathione was markedly inhibited by aluminum in mitochondria when NADP and isocitrate were included in the presence of Mn or Mg; however, addition of excess NADPH completely abolished the effect of aluminum (Fig. 3A). These results suggest that aluminum inhibits GSH regeneration by decreasing the supply of NADPH through the inhibition of NADP-isocitrate dehydrogenase.

We further examined the effect of aluminum ion on the NADP-isocitrate dehydrogenase the only NADPH-generating enzyme in mitochondria. Aluminum ion inhibited mitochondrial NADP-isocitrate dehydrogenase with $I_{0.5}$ values of 2–3 μM when Mn or Mg is used for essential metals (Fig. 3B). This inhibition was well agreed with that of the pig heart mitochondrial NADP-isocitrate dehydrogenase [Yoshino and Murakami, 1992].

Effect of aluminum ion on the regeneration of reduced glutathione from GSSG in the liver cytosol was also analyzed. Regeneration of reduced glutathione from GSSG in the cytosol depends on three types of NADPH-generating reactions, that is, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), malic enzyme, and NADP-isocitrate dehydrogenase. Aluminum did not inhibit the regeneration of GSH when glucose 6-phosphate was used as the substrate for the supply of reduced NADP. Formation of GSH was effectively inhibited under the conditions where malate was used as the substrate for the NADPH-generating enzyme. Furthermore, addition of isocitrate as the substrate for NADPH-generating reaction also caused a

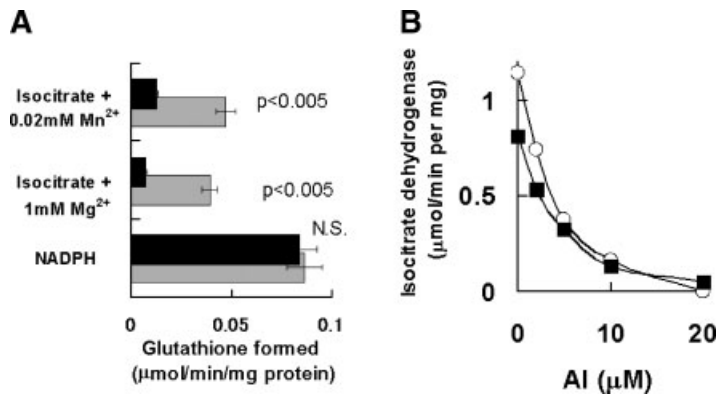

Fig. 3. Effect of aluminum on the formation of reduced glutathione in mitochondria and inhibition of mitochondrial isocitrate dehydrogenase by aluminum ion. **A**: Formation of reduced glutathione in rat liver mitochondria. Reaction mixture of 1 ml contained 50 mM Tris-HCl buffer (pH 7.1), 0.5 mM oxidized glutathione, 0.1 mM DTNB, NADPH-generating system, and mitochondrial fraction in the absence and presence of 0.1 mM aluminum chloride. NADPH-generating system consisted of (a) 0.1 mM threo-DS-isocitrate and 0.1 mM NADP with 0.02 mM $MnCl_2$, and (b) 0.1 mM threo-DS-isocitrate and 0.1 mM NADP with 1 mM $MgCl_2$. ■, 0.1 mM aluminum chloride added; □, no addition. Full activity of GSH formation was obtained by addition of 0.1 mM NADPH. **B**: Inhibition of mitochondrial NADP-isocitrate dehydrogenase by aluminum. Reaction mixture of 1 ml contained 0.1 mM threo-DS-isocitrate, 0.1 mM NADP, 5 μM $MnCl_2$ (○), or 1 mM $MgCl_2$ (■), indicated concentrations of aluminum chloride, and mitochondrial NADP-isocitrate dehydrogenase. Activity was determined by following the change in the absorbance at 340 nm. Data are expressed as mean ± SD, and statistical analyses were performed by Student's *t*-test.

considerable inhibition of regeneration of GSH in cytosol (Fig. 4A).

We explored the effect of aluminum ion on the cytosolic malic enzyme. Aluminum ion inhibited the malic enzyme when Mg^{2+} or Mn^{2+} ion used as the cofactor, but the $I_{0.5}$ values of 20–30 μM were considerably higher than that for mitochondrial NADP-isocitrate dehydrogenase (Fig. 4B).

DISCUSSION

Oxygen is an essential element for the terminal acceptor of the electrons in respiratory chain of aerobic organisms. Molecular oxygen is reduced to water in electron transport system of mitochondria and microsomes. However, this process may produce some reactive oxygen species such as superoxide anion, hydrogen peroxide, and hydroxyl radical as inevitable by-products. Superoxide radical, generated by one-electron reduction of oxygen, is dismuted into hydrogen peroxide by enzymatic and non-enzymatic mechanisms [Fridovich, 1989]. Hydrogen peroxide is further converted to more reactive hydroxyl radical by the Fenton reaction [Fridovich, 1986; Gardner and Fridovich, 1992], which requires reduced iron or copper [Halliwell and Gutteridge, 1990]. Hydroxyl radical, the reactivity of which is markedly high, attacks most molecules found in vivo, and an important antioxidant mechanism is, thus, likely to be the action blocking the formation of hydroxyl radical. In particular, removal of the precursors of reactive oxygen species such as superoxide, hydrogen peroxide and transition metals is prerequisite for antioxidant effects [Halliwell, 1995]. Glutathione peroxidase can act as an antioxidant enzyme scavenging hydrogen peroxide in mitochondria where reactive oxygen species may be easily generated by impairment of electron transport system under the conditions without catalase. Glutathione peroxidase utilizes reduced glutathione to form oxidized glutathione, which should be reduced by glutathione reductase with NADPH. Antioxidant mechanism, thus, depends on the supply of reduced NADP.

Aluminum the most abundant metal in the earth's crust acts as a potent inhibitor of some enzymes in energy metabolism [Yoshino and Murakami, 1992; Yoshino et al., 1992, 1998]. Furthermore, aluminum can enhance the iron-dependent lipid peroxidation of microsomes [Yoshino et al., 1999], and addition of aluminum-maltol complex to HL60 cells induces apoptotic cell death of HL60 cells [Tsubouchi et al., 2001]. Aluminum-dependent cellular toxicity including neurodegenerative disorder can be explained by the prooxidant effect of this metal causing apoptotic cell death. Recently, a mechanism that can explain the prooxidant activity of aluminum was proposed: central to

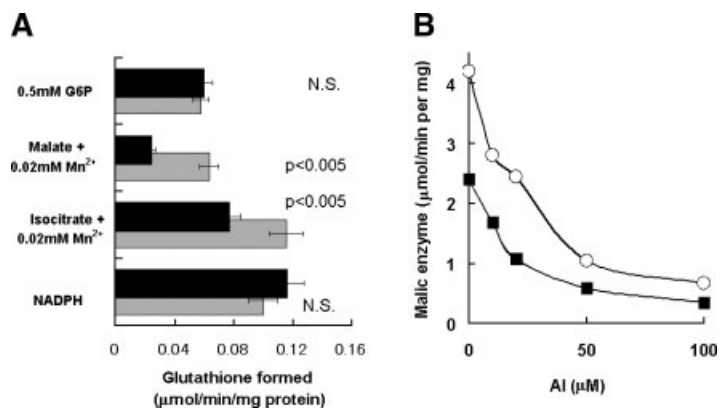

Fig. 4. Effect of aluminum on the formation of reduced glutathione in cytosol and inhibition of cytosolic malic enzyme by aluminum ion. **A**: Formation of reduced glutathione in rat liver cytosol. Reaction mixture of 1 ml contained 50 mM Tris-HCl (pH 7.1), 0.5 mM oxidized glutathione, 0.1 mM DTNB, NADPH-generating system, and cytosolic fraction in the absence and presence of 0.1 mM aluminum chloride. NADPH-generating system consisted of (a) 0.5 mM glucose 6-phosphate with 0.1 mM NADP, (b) 0.5 mM malate and 0.1 mM NADP with 0.02 mM $MnCl_2$, (c) 0.1 mM threo-DS-isocitrate and 0.1 mM NADP with 0.02 mM $MnCl_2$. Full activity of GSH formation was obtained by addition of 0.1 mM NADPH. ■, 0.1 mM aluminum chloride added; ▨, no addition. **B**: Inhibition of cytosolic malic enzyme by aluminum. Reaction mixture of 1 ml contained 50 mM Tris-HCl buffer (pH 7.1), 0.5 mM threo-DS-isocitrate malate, 0.1 mM NADP, 5 mM (○) or 1 mM (■) $MgCl_2$, indicated concentrations of aluminum chloride, and cytosolic malic enzyme. Data are expressed as mean ± SD, and statistical analyses were performed by Student's *t*-test.

the prooxidant mechanism of aluminum is the formation of an aluminum superoxide semiquinone radical [Exley, 2004]. Furthermore, the present study showed that aluminum could inhibit the regeneration of reduced glutathione utilized for scavenging reactive oxygen species, hydrogen peroxide. Aluminum-mediated inhibition of the glutathione regeneration can be explained by decreasing the production of NADPH through the inhibition of NADP-isocitrate dehydrogenase in mitochondria. Aluminum inhibited NADP-isocitrate dehydrogenase with the $I_{0.5}$ value of about 2–3 μM, which is markedly lower than the intracellular aluminum concentration of 20–30 μM [Zatta, 1993], indicating that aluminum can act as a potent inhibitor of mitochondrial NADP-isocitrate dehydrogenase. Reactive oxygen species can be generated from the electron transport system in mitochondria, and NADP-isocitrate dehydrogenase is the only enzyme for the supply of NADPH required for regenerating reduced glutathione as the substrate of glutathione peroxidase in mitochondria [Plaut et al., 1983]. Potent inhibition by aluminum of NADP-isocitrate dehydrogenase may contribute to attenuation of antioxidant system, causing cellular oxidative damage relating to neurodegenerative diseases. On the other hand, aluminum showed only a weak inhibition of cytosolic malic enzyme: the $I_{0.5}$ value of aluminum was tenfold larger than that of NADP-isocitrate dehydrogenase. Considering no inhibition by aluminum of glucose 6-phosphate dehydorgenase, aluminum may show only a little inhibitory effect on the glutathione regeneration in cytosol.

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