Cerebral Oxidative Stress and Mitochondrial Dysfunction in Oxonate-Induced Hyperuricemic Mice

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Relation between blood uric acid levels and brain cell functions were examined using potassium oxonate-induced hyperuricemic mice and allopurinol-induced hypouricemic mice. In hyperuricemic mice and hypouricemic mice, erythrocyte catalase activity was significantly lower and higher, respectively, than that in the control group. No significant change of superoxide dismutase (SOD) activity or glutathione peroxidase activity was observed in either group. Cerebral lipid peroxide levels expressed as thiobarbituric acid reactive substances (TBARS) were significantly higher in hyperuricemic mice, and cerebral mitochondrial ATP synthesis and manganese-containing SOD (Mn-SOD) activity were significantly diminished in the same mice. In hypouricemic mice, no significant change was observed for TBARS levels, mitochondrial ATP synthesis and Mn-SOD activity. Then significant negative correlation between erythrocyte catalase activity and cerebral TBARS levels, and significant positive correlation between catalase activity and mitochondrial ATP synthesis were confirmed. Furthermore, when mouse erythrocytes were treated with uric acid, their catalase activities were particularly diminished. These results suggest that the reduction of erythrocyte catalase activity resulted from the elevation of blood uric acid levels is a major cause of cerebral oxidative stress and mitochondrial dysfunction in hyperuricemic mice. Therefore, it is possible that hyperuricemia and gout are risk factors of cerebral disorders.

Key words —— cerebral oxidative stress, hyperuricemia, mitochondrial dysfunction, catalase, erythrocytes

INTRODUCTION

Catalase plays a major role in cellular antioxidant defense system by decomposing hydrogen peroxide (H₂O₂), thereby preventing the generation of hydroxyl radicals through the Fenton reaction. Consequently, most aerobic cells contain catalase activity.1) Catalase is presented in all major body organs, and is particularly concentrated in liver and erythrocytes, while brain, heart, spleen and skeletal muscle contain lower levels of catalase rather than other tissues.2) Intact erythrocytes are capable of protecting other cells or tissues against several injuries by extracellular H₂O₂.3,4) Since H₂O₂ readily diffuses through membranes, erythrocyte catalase is regarded as a primary enzyme for the removal of extracellular H₂O₂.4,5) Therefore erythrocyte catalase may protect brain, heart, spleen and skeletal muscle against oxidative damage by decomposing H₂O₂. Ho et al. reported that physical impact-induced cortical injury accompanied mitochondrial dysfunction in catalase knockout mice.6) In addition, oxidative stress from catalase deficiency was suggested to contribute to the early development of arteriosclerosis and diabetes mellitus in patients with hypocatalasemia.7,8) Furthermore, in aniridia patients, catalase deficiency is associated with an increased incidence of both mental retardation and the type of cancer known as Wilms’ tumor.9) We recently observed that erythrocyte catalase activity of patients with hyperuricemia was significantly lower than that of healthy subjects. Although hyperuricemia is regarded as a major risk factor of gout, cardiovascular disease and hypertension,10,11) it has been uncertain whether it is related to cerebral disorders, such as cerebrovascular disease, ischemic encephalopathy and neurodegeneration. Since erythrocytes efficiently dispose of extracellular H₂O₂,3,4)
reduction of erythrocyte catalase activity and the resulting increase in $H_2O_2$ levels would cause cerebral disorders in patients with hyperuricemia or gout. In order to evaluate the influence of altered blood uric acid level to erythrocytes and brain cells, we examined the erythrocyte catalase activity, oxidative stress and mitochondrial dysfunction of cerebral cells in both potassium oxonate-induced hyperuricemic mice and allopurinol-induced hypouricemic mice.

**MATERIALS AND METHODS**

**Chemicals** —— Glutathione reductase from bakers yeast (EC 1.6.4.2), catalase from bovine liver (EC 1.11.1.6), glutathione peroxidase (GPx) from bovine erythrocyte (EC 1.11.1.9), xanthine oxidase (XO) from buttermilk (EC 1.1.3.22), reduced glutathione (GSH), digitonin, adenosine 5’-diphosphate (ADP) disodium salt, heparin sodium salt from porcine intestinal mucosa and protease type VIII from Aspergillus saitoi were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Cumene hydroperoxide, allopurinol and nitroblue tetrazolium (NBT) were obtained from Wako Pure Chemical Industries (Osaka, Japan). $O,O’$-Bis (2-aminoethyl) ethyleneglycol-$N,N,N’,N’$-tetraacetic acid (EGTA) was purchased from DOJINDO LABORATORIES (Kumamoto, Japan). Other chemicals of the highest grade were obtained commercially.

**Subjects** —— The subjects enrolled in this study were 20 volunteers composed of 10 patients with hyperuricemia and 10 healthy participants (16 females and 4 males; mean age 39.3 ± 11.3 years) from Keio University Hospital (Tokyo, Japan). Informed consent was obtained from all participating subjects.

**Animal Treatments** —— Male ddY mice (5 weeks of age) purchased from Tokyo Experimental Animal Supply Co. (Tokyo, Japan) were bred with MF pellet basal diet (Oriental Yeast Co., Tokyo, Japan) and tap water for 1 week of acclimation. Mice were housed in an air-conditioned room with temperature of 23 ± 1°C, humidity of 50 ± 3%, and a 12 hr light and dark cycle. Potassium oxonate, an uricase inhibitor, suspended in saline was injected to the abdominal cavity of mice (100 or 300 mg/kg). Similarly, mice were intraperitoneally administered allopurinol, a XO inhibitor, suspended in saline (10 or 50 mg/kg). These administrations were based on the previous papers. The mice were sacrificed 3 hr after the administration. Cerebrum was immediately excised after decapitation, and then weighed, washed with saline and homogenized in ice-cold 0.01 M phosphate buffered saline (PBS) (pH 7.4). The homogenate was used for the measurement of thiobarbituric acid reactive substances (TBARS), mitochondrial respiration and manganese-containing superoxide dismutase (Mn-SOD) activity. Whole blood collected by phlebotomy was used for the measurement of plasma uric acid and erythrocyte antioxidant enzyme activities. For in vitro experiment, erythrocytes were separated from heparinized whole blood of mice. This experimental design was approved by the Animal Experimental Committee of Hoshi University and the mice were cared for in accordance with the Guidelines Concerning the Care and Use of Laboratory Animals.

**Isolation of Cerebral Mitochondria** —— Cerebral mitochondria of mice was isolated by the method of Rosenthal et al. with slight modifications, using 0.02% digitonin to free mitochondria from the synaptosomal fraction. In brief, 5 ml of brain homogenate containing bacterial protease was centrifuged at 2500 rpm for 5 min. The pellet, including the fluffy synaptosomal layer, was resuspended in 2.5 ml of isolation buffer (225 mM mannitol, 75 mM sucrose, 5 mM Hepes, 1 mM EGTA, 1 mg/ml bovine serum albumin (BSA), pH 7.4) containing 0.02% digitonin, and the suspension was centrifuged at 10000 rpm for 10 min. Resulting mitochondrial pellet without the synaptosomal layer was resuspended in 2.5 ml of the isolation buffer and recentrifuged at 10000 rpm for 10 min. The pellet was resuspended in 1 ml of resuspension buffer containing 225 mM mannitol, 75 mM sucrose and 5 mM Hepes (pH 7.4).

**Measurement of Mitochondrial Respiration** —— Oxygen consumption of isolated cerebral mitochondria was polarographically monitored with a Clark oxygen electrode. The reaction was carried out at 37°C in 1.0 ml of the reaction buffer containing 100 mM sucrose, 100 mM KCl, 2 mM KH$_2$PO$_4$, 5 mM Hepes and 10 μM EGTA (pH 7.4) with 0.6 mg mitochondria protein. Oxygen concentration of reaction mixture was continuously measured for 10 min after addition of 75 nmol ADP. Mitochondrial respiration is expressed as decreased O$_2$ nmol/min/mg protein.

**Measurement of Plasma Uric Acid Concentration** —— Measurement of uric acid concentration in deproteinized plasma of mouse blood was carried out by the use of Hitachi HPLC apparatus (pump, L-6000; UV detector, L-4000; chromatointegrator, D-2500; Hitachi Co., Tokyo, Japan) according to the
method of Akaike et al. Detection limit of uric acid in this method was 1 µM or more.

**Measurement of Antioxidant Enzyme Activities** — Cumene hydroperoxide was used as the substrate to eliminate the effect of catalase contamination in the sample solution on the measurement of GPx activity in cerebrum. Briefly, an aliquot of sample solution was mixed with 2.1 ml reaction mixture containing 1 mM EDTA, 0.2 mM NADPH and 1 mM GSH in a phosphate buffer (pH 7.0) and 10 units of glutathione reductase. After pre-incubation, to the reaction mixture, 0.75 µmol of cumene hydroperoxide was added to initiate the enzyme reaction. Reaction was carried out at 25°C in cell holder of spectrophotometer (U-2000; Hitachi Co., Tokyo, Japan) with temperature controller (SDR-30; Hitachi Co.). The absorbance at 340 nm of NADPH in the reaction mixture was continuously recorded. One unit of enzyme catalyzes the oxidation of 1.0 µmol of GSH to oxidized glutathione (GSSG) per min by cumene hydroperoxide.

Catalase activity was measured as described previously. In brief, an aliquot of sample solution was mixed with 3.0 ml phosphate buffer containing 15 mM H₂O₂ (pH 9.0). Reaction was carried out at 25°C in cell holder of spectrophotometer with temperature controller. The absorbance at 240 nm of the reaction mixture was continuously recorded. Enzyme activity was calculated from the decrease in H₂O₂ concentration of reaction mixture using molar extinction coefficient of H₂O₂ at 240 nm (43.6 M⁻¹cm⁻¹). One unit of enzyme decomposes 1.0 µmol of H₂O₂ per min.

Measurement of erythrocyte copper and zinc-containing superoxide dismutase (Cu/Zn-SOD) and cerebral Mn-SOD activities were performed by the method of Beauchamp and Fridovich. Briefly, an aliquot of sample solution was mixed with 2.0 ml reaction mixture containing 0.15 mM EDTA, 0.75 mg BSA, 0.038 mM NBT and 0.15 mM hypoxanthine in a carbonate buffer (pH 10.2). After pre-incubation, in order to initiate the reaction, 1 unit of XO was added and then the absorbance at 560 nm of the reaction mixture was measured after incubation at 25°C for 20 min. One unit of enzyme is defined as the amount of enzyme required for 50% inhibition of the NBT reduction.

**Measurement of Thiobarbituric Acid Reactive Substances** — An aliquot of brain homogenate was heated with thiobarbituric acid for 60 min in boiling water, and the red pigment that appeared was extracted with a mixture of n-butyl alcohol and pyridine (15 : 1; v/v). The absorbance at 535 nm of dehydrated organic solution was measured. The molar extinction coefficient of malondialdehyde at 535 nm (ε = 1.56 × 10² M⁻¹cm⁻¹) was used to calculate TBARS concentration as described elsewhere.

**In Vitro Effect of Uric Acid on Erythrocyte Antioxidant Enzyme Activities** — Erythrocytes suspended in saline were incubated with uric acid at 37°C for 30 min. As shown in Fig. 1, this experiment was carried out at several concentrations of uric acid until 0.5 mM. After the incubation, the reaction mixture was centrifuged at 3000 rpm for 10 min. Pellet was washed twice with saline, subsequently it was hemolyzed with an aliquot of distilled water. Catalase, GPx and Cu/Zn-SOD activities of hemolsates were measured as mentioned above.

**Measurement of Protein Concentration** — Protein concentration in each sample solution was measured by the method of Lowry et al. using BSA as the standard protein.

**Statistics** — Data are expressed as the mean ± S.D. A one-way analysis of variance (ANOVA) was used to determine any significant differences (p < 0.05) between means. When significant differences were found, Duncan’s multiple-range test was used to determine the exact nature of the difference.
RESULTS AND DISCUSSION

In hyperuricemia patients, significant diminution was observed in erythrocyte catalase activity but not in GPx and Cu/Zn-SOD activities (Table 1). In addition, there was significant negative correlation between blood uric acid levels and erythrocyte catalase activity in all subjects ($r = -0.492, p < 0.05$). So, in order to examine the effect of plasma uric acid levels on erythrocyte antioxidant capacity and cerebrum, we measured the erythrocyte antioxidant enzymes and cerebral oxidative stress of hyperuricemic and hypouricemic mice.

In in vivo experiment using potassium oxonate-induced hyperuricemic mice and allopurinol-induced hypouricemic mice, body weight and brain weight was not changed in all experimental groups (data not shown). However, plasma uric acid levels were significantly elevated to approximately 2-fold for the potassium oxonate-induced hyperuricemic mice (Table 2). In contrast, erythrocyte catalase activity was remarkably decreased to approximately 0.6-fold in the same mice. Furthermore, there was the significant negative correlation between catalase activity and uric acid levels of all experimental mice ($r = -0.793; p < 0.01$). Meanwhile, no significant change of erythrocyte Cu/Zn-SOD and GPx activities was observed in the potassium oxonate-treated group. These results indicate that the augmentation of blood uric acid levels specifically caused to decrease in erythrocyte catalase activity. Furthermore physiological level of uric acid (approximately 0.3 mM or more) also inhibited erythrocyte catalase activity but not GPx and Cu/Zn-SOD activities in vitro (Fig. 1). In erythrocytes, catalase is supposed to be a more predominant $H_2O_2$-removing enzyme than GPx and to have an important role in the disposal of exogenous $H_2O_2$, particularly in brain, heart and skeletal muscle. Therefore, it is likely that catalase inhibition and the resulting elevation of extracellular $H_2O_2$ level by increased blood uric acid cause oxidative stress in the tissues of those patients with hyperuricemia or gout. It was reported that there was an influence regarding catalase deficiency to mental retardation in WAGR syndrome and to $H_2O_2$-caused oxidative stress. Thus, decrease in erythrocyte catalase activity might be a cause of cerebral disorders derived from $H_2O_2$ in patients with hyperuricemia or gout.

Erythrocyte catalase activity in the allopurinol-treated group was significantly higher with the lower plasma uric acid levels for those in the control group.
On the other hand, uric acid is also regarded as an antioxidant, being endogenous low-molecular hydroxyl radical scavenger which eliminates the toxic effect of H₂O₂ as catalase and GPx. Therefore, it is our speculation that the elevation of erythrocyte catalase activity may be compensatory action for the decline of blood uric acid levels, and vice versa.

Cerebral TBARS levels, an available parameter of oxidative stress, which increases when reactive oxygen species (ROS) are accumulated, were significantly elevated in the potassium oxonate-treated group (Table 3). Furthermore, significant negative correlation between cerebral TBARS and erythrocyte catalase activity in all experimental mice was observed (Fig. 2A). Since potassium oxonate cannot directly oxidize lipid, protein and other tissue components, the significant increase in TBARS levels in potassium oxonate-treated mice suggests the indirect action of potassium oxonate via the hy-
peruricemia. Although cardiovascular and cerebrovascular diseases are frequently observed in hyperuricemia and gout patients,\(^9,10\) it is uncertain whether they are caused by the elevation of blood uric acid levels. As mentioned above, there was the significant negative correlation between blood uric acid levels and erythrocyte catalase activity. Furthermore, significant positive correlation between blood uric acid levels and cerebral TBARS in all experimental mice was observed (Fig. 2B). Therefore, cerebral oxidative stress seems to be caused by the reduction of erythrocyte catalase activity resulting from the elevation of blood uric acid levels in the potassium oxonate-treated mice. Although uric acid is an endogenous antioxidant,\(^22\) no significant change of cerebral TBARS levels was observed in the allopurinol-treated group (Table 3). It is possible that compensatory increase in erythrocyte catalase activity suppressed cerebral oxidative stress mainly due to \(\text{H}_2\text{O}_2\).

Mitochondrial ATP synthesis is frequently decreased when tissue cells are oxidatively modified.\(^27\) Cerebral mitochondrial ATP synthesis evaluated by the oxygen consumption of both mitochondria and ADP was significantly decreased by the potassium oxonate administration but not by the allopurinol administration (Table 3). Furthermore, there was significant negative correlation between ATP synthesis and blood uric acid levels (Fig. 2C). And significant positive correlation between ATP synthesis and erythrocyte catalase activity was also observed (Fig. 2D). Since erythrocytes have the role to remove extracellular \(\text{H}_2\text{O}_2\),\(^27,28\) brain cells of hyperuricemic mice were likely to be susceptible to oxidative modification by \(\text{H}_2\text{O}_2\), and actually the decrease in ATP synthesis reflects mitochondrial dysfunction. Therefore, it is possible that hyperuricemia and gout patients possess the risk of cerebral disorders resulting from the reduction of erythrocyte catalase activity. Cerebral mitochondrial damage due to the accumulation of ROS was also confirmed by the severe reduction of Mn-SOD activity in the potassium oxonate-treated mice but not in the allopurinol-treated one (Table 3). As shown in Fig. 3A, there was the significant positive correlation between Mn-SOD activity and ATP synthesis. And the significant negative correlation between Mn-SOD activity and cerebral TBARS was also observed (Fig. 3B). Although it is not clear what reduced Mn-SOD activity in potassium oxonate-treated mice, the reduction of Mn-SOD also seems to be critical in cerebral oxidative stress and mitochondrial dysfunction of hyperuricemic mice. Since Mn-SOD knockout mouse was reported to show extensive neurodegeneration,\(^29\) it is possible that both hyperuricemia and gout are neurodegenerative risk factors.

In conclusion, our results suggest that the pathological level of blood uric acid caused by the potassium oxonate administration brought about the reduction of erythrocyte catalase activity, and in turn these aberrations contributed to cerebral oxidative stress and mitochondrial dysfunction. Furthermore, the reduction of Mn-SOD activity by the potassium oxonate administration seems to be a cause of cere-

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**Fig. 3. Correlations between Cerebral Mn-SOD Activity, Mitochondrial ATP Synthesis and TBARS Levels**

(A) Correlation between Mn-SOD activity and mitochondrial ATP synthesis (\(r = 0.691, p < 0.01\)). (B) Correlation between Mn-SOD activity and TBARS levels (\(r = -0.814, p < 0.01\)).
bral oxidative stress and mitochondrial dysfunction. Thus, increase in the uric acid in hyperuricemia and gout is probably toxic for the patients having these disorders.

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