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Increase in Production of Ascorbate Radical in Tissues of Rat Treated with Paraquat

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The production of ascorbate radical ($A^{\bullet-}$) was investigated in tissues of rats intoxicated with paraquat (PQ) to know the protective role of antioxidant ascorbate (AH^-) in tissues. The electron spin resonance (ESR) method is applied to observe $A^{\bullet-}$. To eliminate increased biosynthesis of ascorbic acid (AH_2) by PQ intoxication, ODS rats were chosen and fed with or without 250 ppm PQ in the diet. The radical $A^{\bullet-}$ was detected only in the lung and spleen homogenates of both intoxicated and control rats at the beginning of ESR measurement. The radical levels of intoxicated rat lung and spleen were increased rapidly to twice the initial level after 3 h and decreased to 0.2–0.6 times the initial level after 24 h, whereas those of control rats were increased slowly to 1.1 times the initial level after 4 h and decreased slowly to 0.7 times the initial level after 24 h at 4°C. In other organs such as liver, kidney, heart and testis, $A^{\bullet-}$ was not detected initially but detected afterwards. Higher $A^{\bullet-}$ level was observed in the intoxicated rat liver than the control but no appreciable differences of $A^{\bullet-}$ levels were observed between the intoxicated kidney, heart and testis and the respective controls. In the intoxicated rat lung the concentration of AH_2 is only half but that of $A^{\bullet-}$ is twice as high as that of the control. Larger amounts of $A^{\bullet-}$ produced in the intoxicated rats decayed more quickly than those in the control rats. The simple addition of PQ to the control organ enhanced neither $A^{\bullet-}$ production nor $A^{\bullet-}$ quenching. These facts suggest that the tissues damaged by PQ require larger amounts of AH^- to detoxicate harmful oxidants, resulting in concomitant production of $A^{\bullet-}$.

Keywords: Ascorbate radical, paraquat, ascorbic acid, rat mutant, ESR

INTRODUCTION

The toxicity associated with a herbicide paraquat (PQ) has generally been attributed to the generation of reactive oxygen species by their redox cycling,[1] resulting in oxidative tissue injury. Tissues provide several antioxidants against degeneration caused by this oxidant stress. Ascorbate (AH^-) was reported to be an outstanding antioxidant in human plasma compared with protein thiols, bilirubin, urate and vitamin E.[2] In addition, AH^- is distributed in tissues 50-fold

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as much as in plasma.[3] In rat lung AH^- was more reactive than glutathione in reducing transition metals.[4] When AH^- plays a protective role as a reductant, AH^- itself is oxidized to ascorbate radical ($A^{\bullet-}$) at first, and then to dehydroascorbate.[5] In contrast to the general concept of high reactivity of free radicals, $A^{\bullet-}$ is relatively harmless and nonreactive species which decays mainly by disproportionation, thereby terminating the propagation of free radical reaction.[6] There have been many reports on the relation between $A^{\bullet-}$ in plasma and some diseases[7,8] or toxicosis.[9] Although $A^{\bullet-}$ in tissues has been studied in tumor tissues,[10,11] $A^{\bullet-}$ in tissues after PQ intoxication was not reported before.

To clarify the relation between the PQ intoxication and its detoxication by AH^-, it is desirable to measure $A^{\bullet-}$ level and its time course in respective organs. Since the oxidative damages are influenced by the physiological conditions, and caused by various processes such as metal-ion release, activation of several enzymes, and disruption of electron transport chains leading to mitochondrial deficits, particular attention is needed to establish the relation between PQ intoxication and production of $A^{\bullet-}$. Therefore, our experiment on the production of $A^{\bullet-}$ is designed as follows: (1) ODS rats[12,13] which cannot synthesize AH_2 as humans, are used because the increments of both AH^- and $A^{\bullet-}$ in plasma due to PQ intoxication were observed in ordinary rats which can synthesize AH_2.[9,14] (2) Deferoxamine mesylate (DFO) or EDTA is added before tissue homogenization to eliminate the contribution of free transition metals. (3) The centrifugation is carried out with low (500*g*) and high (16 000*g*) speeds to specify roughly the cellular components which are related to the radical production and quenching. (4) The time course of radical level at 4°C is compared with that at 25°C to estimate the contribution of enzymatic activity. (5) The control tissues spiked with PQ are tested to find out whether the larger radical production is due to merely the presence of PQ itself or the after effects of PQ poisoning.

MATERIALS

Male ODS rats were purchased from Seiken Shizai K.K., Shizuoka, Japan. The composition of basal diet except AH_2 was written in our previous work.[15] The supplementation of AH_2 was 300 ppm and dosing of PQ was 250 ppm in the diet. Twelve rats weighing 160 ± 9 g were divided into two groups; one group consisted of 6 rats that received PQ dosed diet and the other control group consisted of 6 rats that received diet without PQ. The diets and water were given freely. Rats were housed in individual cages in a temperature controlled room (22°C) under 12 h light–dark cycle. They were killed by cardiac puncture under light nembutal anesthesia on day 11. The blood was collected by heparinized syringe, and the plasma was separated by centrifugation. The gross pathological changes of organs were examined at autopsy. The organs such as the lung, spleen, liver, kidney, heart and testis were separated after perfusion with enough amount of ice cold physiological saline through the portal vein. The homogenization was performed with or without 1 mM DFO or EDTA, chelator known to inhibit metal dependent AH^- oxidation.[16] Since we found that neither DFO nor EDTA altered the result, we later used 1 mM DFO added sucrose solution for the homogenization. Namely, one gram of each tissue was homogenized by adding 9 ml (or 19 ml for spleen) of 0.25 M sucrose solution containing 1 mM DFO with a polytron homogenizer (Kinematica, Switzerland) for 2 min with the lowest speed while cooling in ice. The ultra-pure water having specific conductivity of $5.5 \times 10^{-8}\,\Omega^{-1}\,cm^{-1}$ was used for the homogenization. After centrifugation at 500*g* for 2 min or 16 000*g* for 5 min at 4°C, the supernatant of the homogenate was used for the assay of $A^{\bullet-}$.

PQ, DFO and standard materials for spin concentration (4-amino-TEMPO and 3-carboxy-PROXYL) were purchased from Sigma, St. Louis, MO, USA, and other reagents used were of analytical grade.

METHODS

To measure $A^{\bullet-}$ level, a JEOL JES-FE2XG ESR spectrometer was used with a microwave power of 5 mW and a modulation width of 0.1 mT. The spectrometer setting was 328.8 mT: sweep range 5 mT, sweep time 16 min, response time 1 s and gain 10^4 were adopted. A 70 µl aliquot of supernatant of tissue homogenate was put in a quartz flat cell and all measurements were made with the same cell. Electron spin resonance (ESR) spectrum of $A^{\bullet-}$, a characteristic doublet (a hyperfine splitting of 0.18 mT and a g value of 2.0054), was measured at 25°C with a small field (0.5 mT) scan within 1 min after taking out the sample from one of the two containers maintained at either 4°C or 25°C, respectively. Although the temperature of the sample preserved at 4°C may be raised during the ESR measurement at 25°C, no appreciable change in signal intensity was observed in three ESR scans for the same sample. The radical concentration was determined by double integration of the first derivative ESR signal using aqueous solution of either 4-amino-TEMPO or 3-carboxy-PROXYL as a standard. Total AH_2 levels of plasma, lung and liver were measured by using a Shimadzu LC-10AD HPLC as written previously.[9] The PQ concentrations of treated rat organs were measured as reported previously.[9]

The control organ was added with either 50, 5 or 0.5 nmol of PQ per gram wet organ and was homogenized to see whether the increment of radical production in PQ fed rat is due to the effect of PQ poisoning or merely the coexistence of PQ itself.

RESULTS

The rats fed with PQ began to display several symptoms such as anorexia, hypokinesia, diarrhea, epistaxis, tremor and their pili became rough after 7 days. The intoxicated and healthy rats were killed on day 11. Severe hemorrhage and congestion of lung were observed in PQ fed rats.

The speed of centrifugation of tissue homogenates was varied from 500g to 16 000g, to specify the cellular components which were related to radical production and quenching. Since the time course of $A^{\bullet-}$ level varied gradually with the speed of centrifugals, two extreme speeds, i.e., 500g for 2 min and 16 000g for 5 min, were chosen and the results are shown in Figure 1. At 16 000g $A^{\bullet-}$ in the supernatant of the control rat lung seems to consist of one component which is detectable at the beginning of measurement, and is stable for 8 h. The behavior is similar to that of $A^{\bullet-}$ in control plasma indicated in Table I, which is detectable just after the centrifugation of blood and stable for a long time as reported previously.[9] At 500g $A^{\bullet-}$ in the supernatant seems to consist of two components; first component, say, plasma-type, is that found in 16 000g supernatant and the second component, say, cell-type, is that which is not detectable initially but increases afterwards, and decreases quickly. Judging from the centrifugation speed, the cell-type might be related with mitochondria or with larger particles than mitochondria. Comparing with results of two speeds of centrifugation, the centrifugation at 500g is adopted for all organs

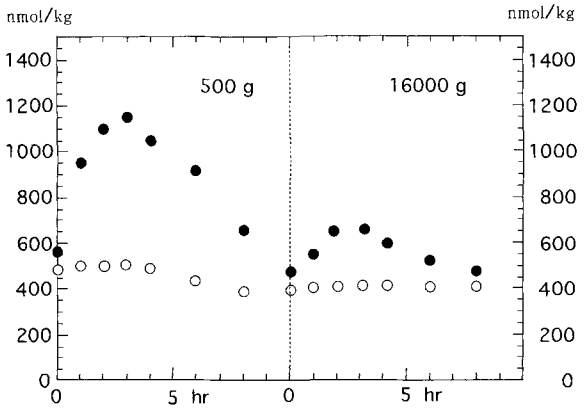

FIGURE 1 The effect of centrifugation speed on time course of ascorbate radical level in lung homogenate preserved at 4°C. ● and ○ indicate the radical level of PQ fed rat and control rat, respectively. The left side and right side of the figure show the level in the supernatant centrifuged at 500g for 2 min and that in the supernatant obtained separately at 16 000g for 5 min from the same homogenate.

TABLE I Time courses of ascorbate radical ($A^{\bullet-}$) level in plasma and organ homogenates preserved at 4°C. The initial level, the maximum level and the level after 24 h, and the time which shows the maximum are indicated. $A^{\bullet-}$ level is expressed as nmol/l in plasma and nmol/kg in organ. The number of rats is six for each group

Organs	+PQ (+) Control (−)	$A^{\bullet-}_{initial}$ mean ± SD	$A^{\bullet-}_{max}$ mean ± SD	$A^{\bullet-}_{24h}$ mean ± SD	Time-max (h)
Plasma	+(n.d.)	31 ± 5	31 ± 5	25 ± 5	0
Plasma	−	28 ± 4	28 ± 4	25 ± 4	0
Lung	750 ± 220	560 ± 50	1160 ± 150**	250 ± 70	3
Lung	−	480 ± 50	510 ± 50	300 ± 50	3
Spleen	220 (mix)	1200 ± 150	2440 ± 490**	800 ± 150	3
Spleen	−	1100 ± 150	1200 ± 150	900 ± 150	4
Liver	50 (mix)	0	560 ± 80**	150 ± 40	2
Liver	−	0	340 ± 60	200 ± 60	3
Kidney	380 ± 110	0	260 ± 50	0	2
Kidney	−	0	260 ± 50	0	3
Heart	50 (mix)	0	350 ± 80	140 ± 40	5
Heart	−	0	320 ± 60	190 ± 50	6
Testis	50 (mix)	0	360 ± 80	360 ± 80	24
Testis	−	0	340 ± 80	340 ± 80	24

The detection limit of $A^{\bullet-}$ is 4 nmol/l for plasma, 80 nmol/kg for spleen and 40 nmol/kg for the other five organs. PQ concentration is expressed as nmol/kg organ. (n.d.) and (mix) indicate the value of PQ not detected and the value obtained by the mixed samples of six rats, respectively.
** Significantly different ($P < 0.001$) from the control.

because the difference between the intoxicated and the control is larger, and the signal itself is larger in 500*g* supernatant than that in 16 000*g* supernatant.

The time courses of $A^{\bullet-}$ in several tissue homogenates preserved at 4°C are shown in Table I, which summarizes the initial and the maximum $A^{\bullet-}$ levels as well as those after 24 h, and the time which exhibits the maximum. Only $A^{\bullet-}$ was observed at around $g = 2.0054$ in all tissue homogenates. Like lung, spleen of both intoxicated and control rats exhibited $A^{\bullet-}$ at the beginning of measurement, and their $A^{\bullet-}$ consists of two types as shown in Figure 2. The radical levels in both lung and spleen of intoxicated rats increased rapidly to twice the initial level after 3 h and decreased to about 0.2–0.6 times the initial level after 24 h as indicated in Table I. The radical levels in both lung and spleen of the control rats increased slowly to 1.1 times the initial level after 4 h and decreased slowly to about 0.7 times the initial level after 24 h.

In liver, kidney, heart and testis, $A^{\bullet-}$ could not be detected initially but was detected later, and the $A^{\bullet-}$ seems to consist of only the cell-type

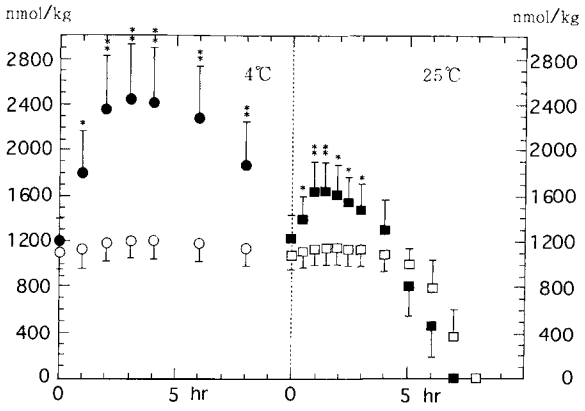

FIGURE 2 The effect of preserving temperature on time course of ascorbate radical level in spleen homogenates centrifuged at 500*g* for 2 min. ● and ○ indicate the radical levels preserved at 4°C of six rats fed with PQ and six control rats, respectively; ■ and □, those preserved at 25°C of six rats fed with PQ and six control rats, respectively. The error bar represents the mean ± SD. * and ** indicate that *P* value is less than 0.05 and 0.001 respectively.

found in lung. As an example, Figure 3 shows the time course of $A^{\bullet-}$ in liver homogenates. The radical levels of both intoxicated and control rats attained their maximum rapidly after 2 h and the maximum level of the intoxicated liver was

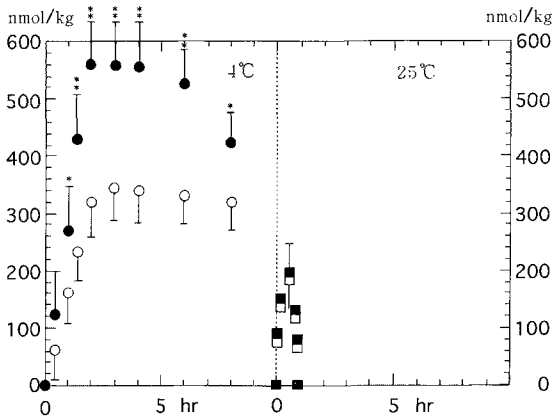

FIGURE 3 The effect of preserving temperature on time course of ascorbate radical level in liver homogenates centrifuged at 500*g* for 2 min. Symbols are the same as those in Figure 2.

nearly twice that of the control. These results of the time courses of $A^{\bullet-}$ levels in Table I indicate that the time courses in respective organs were different from one another. A significant difference in $A^{\bullet-}$ levels between the intoxicated and the control was observed in lung, spleen and liver.

The effect of preserving temperature was compared between 4 and 25°C. When the homogenates of lung and spleen were kept at 25°C, the maximum radical level of the intoxicated rat was attained within 1 h and that of the control rat at 2 h, and both levels became zero within 8 h. The results of spleen are shown in Figure 2. In liver and kidney $A^{\bullet-}$ could be detected only within 40 min at 25°C. The results of liver are shown in Figure 3 which indicate that not the production but quenching of the radical is accelerated by raising the temperature.

Since organs of treated rats contain PQ, the effect of *in vitro* addition of PQ on $A^{\bullet-}$ production and quenching was examined. The PQ concentration in the treated rat organs was less than 0.75 μmol/kg organ as listed in Table I. The addition of either 50, 5 or 0.5 μmol of PQ/kg of the control organ had no effect on the time courses of $A^{\bullet-}$ level in the organ homogenates. Therefore, the increment of $A^{\bullet-}$ production in the rats fed with PQ may be derived from the tissue injury due to PQ poisoning.

The total AH_2 levels in plasma, lung and liver of six PQ fed rats were 27.4 ± 1.5 μmol/l, 1270 ± 310 and 1040 ± 130 μmol/kg respectively, whereas those of six control rats were 35.0 ± 2.8 μmol/l, 2330 ± 100 and 1240 ± 200 μmol/kg respectively, indicating the depletion of AH_2 by PQ treatment.

DISCUSSION

It is known that PQ enhances production of reactive oxygen species such as O_2^-, H_2O_2, $OH^\bullet$, peroxyl radical and alkoxyl radical, although these oxidants are produced continuously as by-products of aerobic metabolism.[1,17] Several protective enzymes such as superoxide dismutase, peroxidase and catalase are activated to detoxicate these oxidants. The reduction of the oxidants by these enzymes results in the oxidation of AH^- to $A^{\bullet-}$.[18] The dopamine-β-hydroxylase is also known to produce $A^{\bullet-}$.[19] When the defense systems in cells have some imperfections, cellular macromolecules such as DNA, protein and lipid are oxidatively damaged[17] and in some cases, transition metals are released from binding proteins.[20] These damaged molecules lead to an increase in production of oxidants.[17] Since the redox potential of $A^{\bullet-}$ formation is the lowest among the redox potentials of the above-mentioned oxidants,[16] $A^{\bullet-}$ is supposed to be produced when damaged tissues produce oxidants. In the present work, larger production of $A^{\bullet-}$ is observed in the tissue homogenates of PQ fed rats than in those of the control rats. This enhancement is not induced by *in vitro* addition of PQ to the control organs as mentioned in the previous section, indicating that some chemical reactions are responsible for the radical production in the tissues already injured by PQ poisoning. Also it is not due to the reaction with free transition metals released from the damaged macromolecules, because neither DFO nor EDTA altered the results. At present, however, the cause

of the larger $A^{\bullet-}$ production in PQ fed rats cannot be specified whether it is due to larger production of oxidants or due to activation of some enzymes which produce $A^{\bullet-}$.

Concerning organ specificity, lung, spleen and liver of treated rats exhibited enhanced production of $A^{\bullet-}$, that is, the maximum level of these rats was twice that of the respective controls. Such high levels are conceivable because PQ damages lung mainly and is metabolized in liver, although we cannot explain at present why the spleen of treated rats showed the higher radical level than the control. As written in the previous section, AH_2 level of plasma, lung or liver of ODS rats fed with PQ was lower than that of the respective controls, indicating that AH_2 had been consumed more significantly in rats fed with PQ than the control. The depletion of AH_2 due to oxidative stress is common in humans.[7,8] In spite of their AH_2 depletion, both lung and liver of treated rats showed higher radical levels than the control. The lung contains ascorbic acid not only intracellularly but also extracellularly in airspace fluid.[21] This may be one of the reasons why two types of $A^{\bullet-}$ are detected in lung. At the beginning of the measurement, $A^{\bullet-}$ originated from AH^- in airspace fluid may be detected and correspond to the plasma-type $A^{\bullet-}$ in lung, which shows the similar behaviors to those of $A^{\bullet-}$ in plasma.

The $A^{\bullet-}$ is known to be quenched by disproportionation.[6] That is, two molecules of $A^{\bullet-}$ yield one reduced form, AH^-, and one oxidized form, dehydroascorbate. Some enzymes such as NADH $A^{\bullet-}$ reductase[19] and semidehydroascorbate reductase[5] are known to quench $A^{\bullet-}$. If the radical disproportionation had proceeded without enzymes, the same decay speeds would be expected for the same amounts of $A^{\bullet-}$ in solutions with nearly the same pH, irrespective of the organ as well as the occurrence of injury. As shown in Table I, the decay speed of the same amount of $A^{\bullet-}$ varies from organ to organ and the decay speed in injured organ is higher than that in the control. The higher initial $A^{\bullet-}$ levels followed by their more rapid disappearances than the respective control were also observed previously in rat and human plasma intoxicated with PQ,[9] and in human plasma after open-heart surgery,[7] and in human plasma from women with preeclampsia.[8] These observations on the radical decay suggest that some enzymes which quench the radical are contained more abundantly in injured organs and plasma. Semidehydroascorbate reductase may be one of the candidates because it is contained most in kidney and secondly in liver,[5] and the radical decay speed is high in these organs. When the temperature was raised from 4 to 25°C, the quenching of $A^{\bullet-}$ was so accelerated that the radical steady state could not be observed in these organs, as shown in Figure 3.

The procedure of the present measurement using ESR is simple and the results are reproducible, since no other signals appeared in the measured region of the field, and the maximum $A^{\bullet-}$ level lasted at least for 1 h at 4°C. Therefore, $A^{\bullet-}$ signal can be used as a convenient tool for monitoring AH^- oxidation in other toxicosis, disease and injury, though the role of $A^{\bullet-}$ even in plasma is still unknown.

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