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原　　報

TISSUE DAMAGE BY DIQUAT REVEALED BY ASCORBATE FREE RADICAL FORMATION

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アスコルベートラジカル産生からみたジクワットによる組織の損傷

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Summary

In vitro formation of ascorbate radical ($A^{\cdot-}$) has been demonstrated for the supernatant fractions of homogenates of rat spleen, lung, kidney, liver, heart and testis after chronic administration of diquat (DQ) to the animals. Male ODS rats, unable to synthesize ascorbic acid (AH_2), were fed with or without 587 ppm DQ in the diet for 14 days. The supernatants of the tissue homogenates were kept at 4℃ for various intervals, and their $A^{\cdot-}$ levels were measured by an electron spin resonance (ESR) method. At the beginning of the measurements, $A^{\cdot-}$ was detected only in the supernatant fractions of the spleen and lung of both untreated and treated rats. In the spleen supernatant of the treated rat, $A^{\cdot-}$ level increased to twice the initial level at 2 h and decreased to 0.2−0.6-fold of the initial level at 24 h, whereas that in control rat spleen supernatant increased slowly to 1.1 times the initial level at 3 h and decreased to 0.7-fold at 24 h. The maximum $A^{\cdot-}$ level in the treated rat lung supernatant was only 1.2 times that of the control. In the supernatant fractions of other organs of both untreated and treated rats, such as the kidney, liver, heart and testis, $A^{\cdot-}$ was not detected initially but became detectable according to time; in the treated kidney and liver supernatant fractions, the maximum $A^{\cdot-}$ levels were nearly twice those of controls, like in the spleen homogenate. For heart and testis homogenates, no significant differences were observed between test and control samples. The level of DQ, known to be metabolized in the liver, was highest in the spleen followed by the kidney. Direct addition of DQ to control organ homogenates, however, neither enhanced nor quenched $A^{\cdot-}$ formation. Therefore, the formation of $A^{\cdot-}$ in tissue supernatant of the treated rats may be due to induction of $A^{\cdot-}$ forming oxidative enzyme(s) and/or due to formation of reactive oxygen species during chronic exposure of the animals to DQ. Our results also indicate that $A^{\cdot-}$ is one of the good indicators of tissue damages.

Key words: Diquat; Ascorbate radical; Electron spin resonance; ODS Rat

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Introduction

Ascorbate (AH^-) has an essential role in the protection of host tissues and body fluids against oxidative attacks [1]. In this process AH^- is oxidized in two steps to yield ascorbate radical ($A^{\cdot-}$) at first and then dehydroascorbate [2]. The signal intensity of $A^{\cdot-}$ is dependent on pH, catalytic metal concentration, oxygen concentration and AH^- concentration. When these variables are well controlled, the steady-state $A^{\cdot-}$ signal may serve as a marker for the degree of free radical oxidative stress in tissues. In tissues intoxicated with diquat (DQ), $A^{\cdot-}$ has not been studied before, although $A^{\cdot-}$ in tumor tissues [3,4] and tissues intoxicated with paraquat (PQ) [5] was examined. PQ and DQ show different toxic effects in mammals, despite their quite similar chemical and herbicidal effects. For example, PQ causes a serious damage to the lung, whereas DQ does not. When equimolar amounts of PQ and DQ were ingested, the molar ratio PQ/DQ contained in tissues of patients varied from 0.08 to 8 [6]. In our previous work [7], the increments of both ascorbic acid (AH_2) and $A^{\cdot-}$ due to DQ intoxication were observed in ordinary rats which can synthesize AH_2. To avoid the effect of its biosynthesis in this study, we have used ODS rats which cannot synthesize AH_2 like humans [8].

Experimental

Male ODS rats were purchased from Seiken Shizai K. K., Shizuoka, Japan. The composition of basal diet except AH_2 was described in our previous report [5]. The supplementation of AH_2 was 1000 ppm. No deaths had been reported by Clark and Hurst [9] for specific-pathogen-free rats of the Alderley Park strain weighing 180—200 g fed with a diet containing 1000 ppm DQ dichloride (i.e., 782 ppm DQ) for two years. Therefore, 587 ppm of DQ was chosen for the present diet, which is equivalent to about 75% of the level of Clark and Hurst [9]. The test group (n=7) received DQ-dosed diet and the control group (n=7) received the same diet without DQ. The diets and water were given freely. Rats were housed in individual cages in a temperature-controlled room (22℃) under 12 h light-dark cycle. They were killed by cardiac puncture under light nembutal anesthesia on day 14. The gross pathological changes of organs were examined at autopsy. The organs such as the spleen, lung, kidney, liver, heart and testis were separated after perfusion with an enough amount of ice-cold physiological saline through the portal vein. One gram of each tissue was placed in 9 ml (or 19 ml for the spleen) of 0.25 M sucrose solution containing 1 mM deferoxamine mesylate (DFO), and homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) for 2 min with the lowest speed under cooling in ice. After centrifugation at 500 g for 2 min at 4℃, the supernatant of the homogenate was kept at 4℃ for various intervals, and used for the assay of $A^{\cdot-}$.

DQ dibromide monohydrate was kindly donated by Zeneca K. K. Agrochemicals, Tokyo, Japan and DFO were purchased from Sigma, St. Louis, MO, USA, and other reagents used were of analytical grade. The ultra-pure water having specific conductivity of $5.5\times10^{-8}\Omega^{-1}$ cm^{-1} containing 1 mM DFO, a chelator known to inhibit metal dependent AH^-oxidation, was used for homogenization of tissues, since autoxidation of AH^- can be avoided at pH below 7 by chelation of catalytic metals [10].

To measure $A^{\cdot-}$ level, a JEOL JES-FE2XG ESR spectrometer was used with a microwave power of 5 mW and a modulation width of 1 gauss. The spectrometer setting was 3288 gauss; a sweep range, 50 gauss; sweep time, 16 min; response time, 1 s; and gain, 10^4. The supernatants (70 μl) of tissue homogenates were placed in a quartz flat cell and the cell was placed in an ESR cavity. An $A^{\cdot-}$ signal with a g value of 2.0054 and a hyperfine splitting of 1.80 gauss was obtained with a small field (5 gauss) scan. The radical levels were measured with peak heights of the signals as described in our previous work [7]. The measurements of $A^{\cdot-}$ levels were made at 25℃ within 1 min after taking out the sample from a container maintained at 4℃. The DQ concentrations of the treated rat organs were measured, after the $A^{\cdot-}$ measurements, as reported previously [7].

The control tissue was spiked with either 10, 1 or 0.1 μg DQ/g and was homogenized to test whether the increment of radical production in the DQ-fed rats is due to the vital reactions of cells against toxic DQ or merely due to the presence of DQ itself.

Results

Food intake was measured every day. The mean food intake of the DQ-fed rat was 5 g/day, whereas that of control rat was 12 g/day. The DQ-fed rats started diarrhea from day 4; their hair became rough but they moved actively as control rats, while rats fed with 250 ppm PQ dichloride showed extra-symptoms such as hypokinesia, epistaxis and tremor [5]. The DQ-fed and control rats were killed on day 14. Some swelling of the kidney was observed but no appreciable changes were observed in the lung, spleen, liver, heart and testis of the DQ-fed rats. In the DQ-fed rats, the ratio of kidney-weight per body-weight was 1.2 times and the ratio of liver-weight per body-weight was 0.8-fold of that of the control, respectively, showing that especially the liver weight was significantly decreased by the DQ treatment.

Only $A^{\cdot-}$ signal was observed at around g=2.0054 in all tissue homogenates. The peak $A^{\cdot-}$ level was highest in the spleen, followed by the lung, liver, kidney, testis and heart of DQ-fed rats as shown in Figs. 1 to 6. The $A^{\cdot-}$ level in spleen supernatant of the treated rats increased to twice the initial level after 3 h and decreased to about 0.2–0.6-fold of the initial level after 24 h at 4℃ as shown in Fig. 1. The maximum $A^{\cdot-}$ level in the lung of the treated rats was only

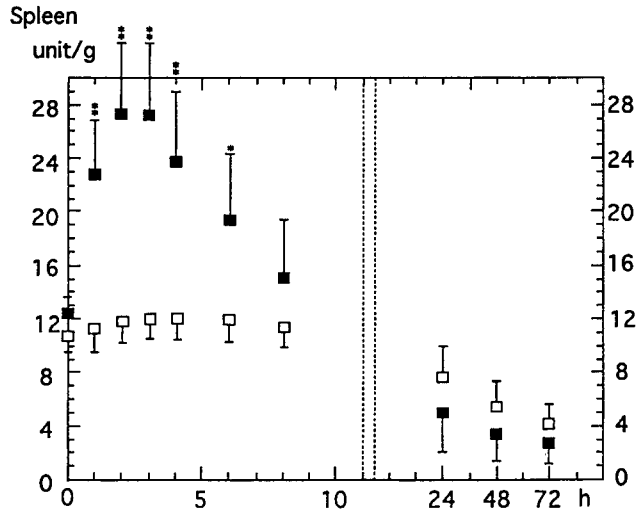

Fig. 1. Time course of ascorbate radical levels in the supernatant fractions of spleen homogenates kept a 4℃. DQ-treated rat, ■, control rat, □, with the mean±SD. *and** indicate that P value is less than 0.05 and 0.001, respectively.

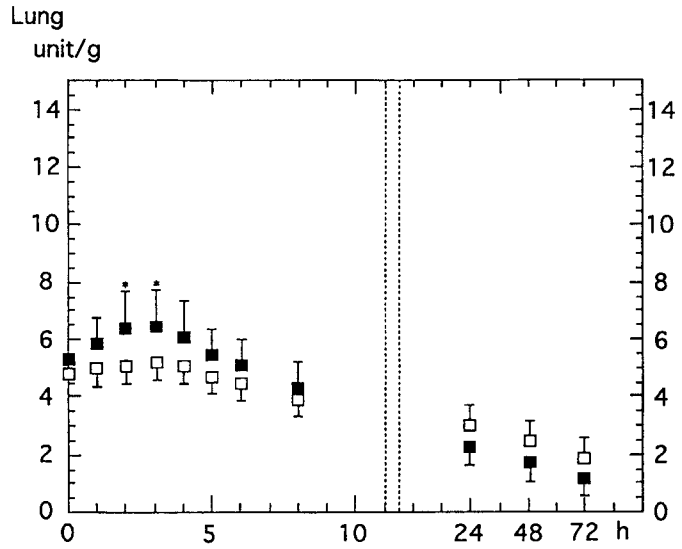

Fig. 2. Time courses of ascorbate radical levels in the supernatant fractions of lung homogenates kept at 4℃. Symbols are the same as in Fig. 1.

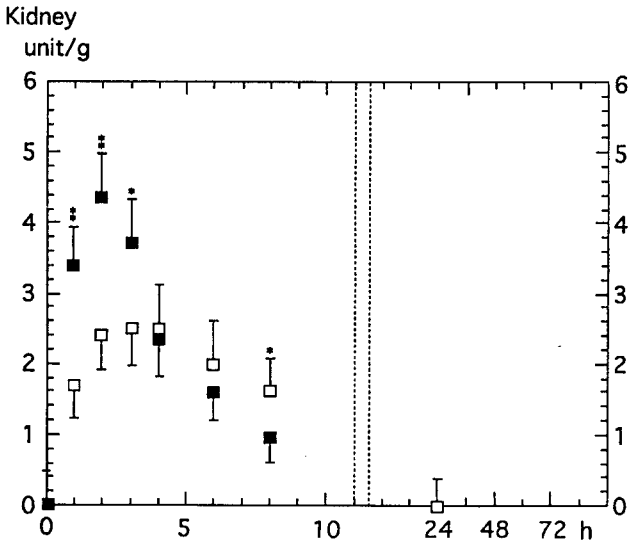

Fig. 3. Time courses of ascorbate radical levels in the supernatant fractions of kidney homogenates kept at 4℃. Symbols are the same as in Fig. 1.

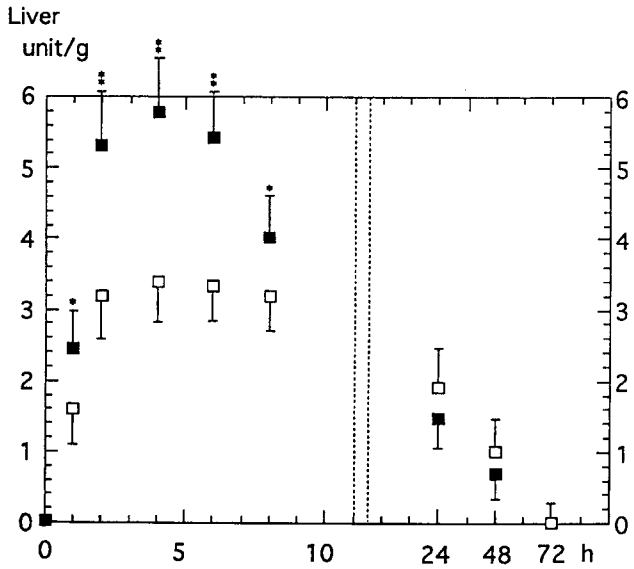

Fig. 4. Time courses of ascorbate radical levels in the supernatant fractions of liver homogenates kept at 4℃. Symbols are the same as in Fig. 1.

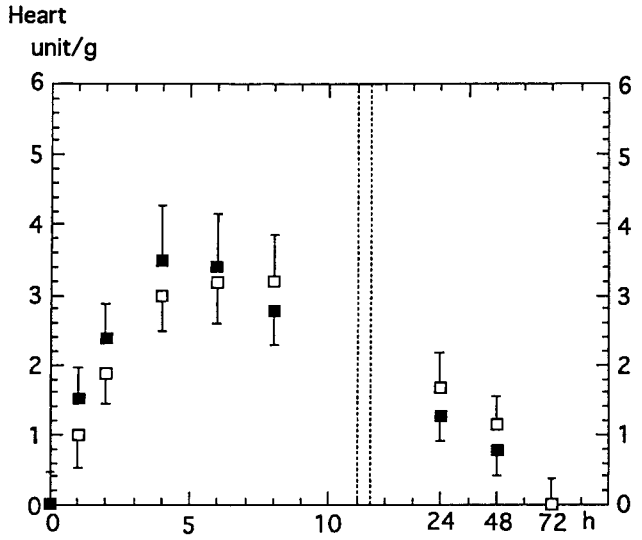

Fig. 5. Time courses of ascorbate radical levels in the supernatant fractions of heart homogenates kept at 4℃. Symbols are the same as in Fig. 1.

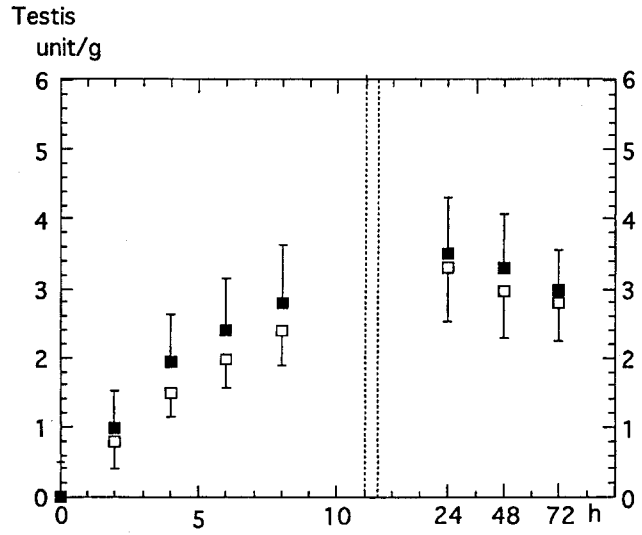

Fig. 6. Time courses of ascorbate radical levels in the supernatant fractions of testis homogenates kept at 4℃. Symbols are the same as in Fig. 1.

1.2 times that of the control as shown in Fig. 2, indicating the influence of DQ on the lung was less than that on the spleen. The $A^{\cdot-}$ levels in both spleen and lung supernatants of control rats increased slowly to 1.1 times the initial level after 4 h and decreased slowly to about 0.7-fold of the initial level after 24 h.

In the supernatant fractions of the kidney, liver, heart and testis, $A^{\cdot-}$ could not be detected

initially for both treated and untreated animals, but was detected later as shown in Figs. 3 to 6. The maximum levels in the supernatants of the kidney and liver of the treated animals were nearly twice those of the control. The $A^{\cdot-}$ was produced and quenched quickly in the kidney supernatant, and very slowly in the testis supernatant. These results indicate that the time courses of $A^{\cdot-}$ levels vary from organ to organ even in control rats.

The DQ contents were 0.38±0.08 and 0.33±0.06 μg/g for the spleen and kidney of 7 treated rats, respectively, and less than 0.02 μg/g for either lung, liver, heart or testis. The *in vitro* addition of either 0.1, 1 or 10 μg of DQ to 1 g of the control tissues had no effects on the time courses of $A^{\cdot-}$ levels in the supernatant fractions of the homogenates.

Discussion

In the present study, chronic DQ administration to ODS rats resulted in enhanced formation of $A^{\cdot-}$ in the supernatant fractions of the spleen, lung, kidney and liver (Figs. 1—4), while *in vitro* direct addition of DQ to control tissue homogenates had no effects on $A^{\cdot-}$. Oxidation of AH^- to $A^{\cdot-}$ is facilitated by interactions with either transition metals, reactive oxygen species [10], and/or oxidative enzymes [10]. The addition of DFO did not affect the results, suggesting that free transition metals, which may be released from injured tissues, made no contribution to the present AH^- oxidation. In the damaged tissues, reactive oxygen species, such as hydroxyl, peroxyl and alkoxyl radicals, are produced more frequently [10]. This may result in concomitant formation of $A^{\cdot-}$ in damaged tissues than in control tissues, since the redox potential is lowest for $A^{\cdot-}$ among reactive oxygen species [10]. Another possibility is the induction of oxidative enzymes, which is related to $A^{\cdot-}$ formation, during chronic exposure of animals to DQ.

It is known that $A^{\cdot-}$ is reduced by NADH-$A^{\cdot-}$ reductase [11] and semidehydroascorbate reductase [2]; it is further oxidized to dehydroascorbate [2], or decomposed by disproportionation [12]. The semideydroascorbate reductase may be related to the quenching, because the radical quenching speed is highest in the kidney, followed by the liver; this enzyme is contained most abundantly in the kidney and secondly in the liver [2]. As we reported previously, the change of $A^{\cdot-}$ level in control plasma at 4℃ was very slow [5], which suggests that decomposition of $A^{\cdot-}$ by disproportionation is unlikely.

A large dose of DQ gave rise to symptoms indicative of its action on the central nervous system, but smaller dose, though lethal, did not give an obvious symptoms to account for death [9]. In the rats receiving diet containing 782 ppm DQ for two years [9], haematological examination, analysis of urine, and gross and microscopic pathological examination did not reveal any changes except for cataract in the eye. Likewise, major organs did not reveal any lesions at the present chronic dose. The maximum levels of $A^{\cdot-}$ in the supernatant fractions of the spleen, kidney and liver intoxicated with DQ, however, were twice those of the control.

Figs. 1, 3 and 4 indicate that $A^{\cdot-}$ is one of good indicators of tissue injury. Since ESR is a non-destructive analytical technique, tissue homogenate after measurements of $A^{\cdot-}$ can be used again for analysis of some acute phase reactant proteins [7] as well as quantification of PQ and DQ.

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References

1) Frei, B. , England, L. and Ames, B.N.: Ascorbate is an outstanding antioxidant in human blood plasma. Proc Natl Acad Sci USA, **86**, 6377−6381 (1989).

2) Diliberto, E.J., Dean, J.G., Carter C. and Allen P.L.: Tissue, subcellular, and submitochondrial distributions of semidehydroascorbate reductase: possible role of semidehydroascorbate reductase in cofactor regeneration. J Neurochem, **39**, 563−568 (1982).

3) Dodd, N.C.F.: Some EPR signals in tumor tissue. Brit J Cancer, **28**, 257−262 (1973).

4) Duke, P.S.: Relation of melanoma homogenate and ascorbate solution. Electron paramagnetic resonance doublets. Exp Mol Pathol, **8**, 112−122 (1968).

5) Minakata, K., Suzuki, O., Horio, H., Saito, S. and Harada, N.: Increase in production of ascorbate radical in tissues of rat treated with paraquat. Free Rad Res, in press.

6) Ameno, K., Fuke, C., Shirakawa, Y., Ogura,S., Ameno, S., Kiriu, T., Kinoshita, H. and Ijiri, I.: Different distribution of paraquat and diquat in human poisoning cases after ingestion of a combined herbicide. Arch Toxicol, **68**, 134−137 (1994).

7) Minakata, K., Suzuki, O., Saito S. and Harada N.: Diquat increases cysteine proteinase inhibitor greatly in rat plasma and tissues. Arch Toxicol, **69**, 318−321 (1995).

8) Horio, F., Ozaki, K., Kohmura, M., Yoshida, A., Makino S. and Hayashi, Y.: Ascorbic acid requirement for the induction of microsomal drug-metabolizing enzymes in a rat mutant unable to synthesize ascorbic acid. J Nutr, **116**, 2278−2289 (1986).

9) Clark, D.G. and Hurst, E.W. : The toxicity of diquat. Brit J Ind Med, **27**, 51−55 (1970).

10) Buettner, G.R. and Jurkiewicz, B.A.: Ascorbate free radical as a marker of oxidative stress: an EPR study. Free Rad Biol Med, **14**, 49−55 (1993).

11) Navas, P., Villalba, J.M. and Cordoba, F.: Ascorbate function at the plasma membrane. Biochim Biophys Acta, **1197**, 1−13 (1994).

12) Bielski, B.H.J., Richter, H.W. and Chan, P.C.: Some properties of the ascorbate free radical. Ann New York Acad Sci, **258**, 231−237 (1975).