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Normalizing renal reducing ability prevents adriamycin-induced proteinuria

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Abstract

Reactive oxygen species play an important role in adriamycin (ADR) nephropathy. We showed by in vivo electron paramagnetic resonance (EPR) that renal reducing ability (RRA) declined on the 7th day after ADR administration. Proteinuria appeared after the decline in RRA. The aim of this study was to prove by in vivo EPR whether the decline in RRA is altered by scavengers such as dimethyl sulfoxide (DMSO) and dimethylthiourea (DMTU) and that it is this change which is responsible for the proteinuria in ADR nephropathy. By showing that DMSO and DMTU ameliorate the RRA, we demonstrate that the decline in RRA is related to ADR-induced proteinuria.

Keywords: Adriamycin nephropathy; Reactive oxygen species; Antioxidants; Renal reducing ability; Electron paramagnetic resonance

Reactive oxygen species (ROS) have been suggested to play an important role in adriamycin (ADR) nephropathy. However, due to the short life of ROS in biological systems, it is difficult to evaluate its in vivo direct participation in the pathogenesis.

Electron paramagnetic resonance (EPR) spectroscopy is a unique technique to detect electron spins directly. In vivo anti-oxidant ability (i.e., reducing ability) is influenced by a balance between the amount of oxidant and reductant. ROS mainly functions as an oxidant, so it is generally thought that ADR administration impaired the renal reducing ability (RRA). By using an EPR technique, in vivo reducing ability can be estimated. Nitroxide radical is a relatively stable free radical. Its electron spin is located at the nitrosyl group. The stability of the nitrosyl group itself originates from the strong three-electron nitrogen–oxygen bond. Furthermore, the tetramethyl group protects the nitrosyl group from attacks by other molecules in biological samples [1]. However, nitroxide radicals are reduced to EPR silent compounds by various reactions because the reduction potential of nitroxide is relatively low [2]. The effect of other reactions, other than reductions, on the metabolism of nitroxide radicals is negligible. It is thought that the reduction sites are the respiratory chain in the mitochondria [3], microsome [4], and several antioxidants in the cytosol [5]. The decay rate of nitroxide radical signal intensity obtained in the kidneys is indicative of RRA.

Dimethyl sulfoxide (DMSO) and dimethylthiourea (DMTU) are scavengers of hydroxyl radicals. It was reported that DMSO, DMTU-treated groups had a significant suppression of urinary protein excretion compared to ADR group [6,7]. Although, as shown in these reports, DMSO and DMTU have the effect of decreasing urinary protein in ADR nephropathy, the relation between the

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antioxidant effect of these drugs and proteinuria has never been proven directly in vivo.

We have already succeeded in measuring the temporal changes in EPR signal intensities in ADR nephropathy at the kidney of a living rat after the administration of a nitroxide radical, 4-hydroxyl 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) [8]. It was noted that the RRA declined on the 7th day after the ADR administration and thereafter continuous urinary protein appeared. The purpose of the present work was to study whether DMSO and DMTU act as the antioxidant directly in vivo in rats and the resulting impaired RRA is associated with ADR-induced proteinuria.

Materials and methods

Chemicals. ADR was purchased from Wako Pure Chemical Industries (Osaka, Japan). TEMPOL, DMTU were obtained from Sigma Chemical (St. Louis, MO). DMSO was purchased from Kanto Chemical (Tokyo, Japan). All other chemicals and reagents were of analytical grade.

Animals and basic procedures. Male Wistar rats (180–200 g body weight) were given free access to standard laboratory chow and water. The rats were divided into six groups. Rats in group A were administered 5 mg/kg b.wt. ADR dissolved in 1 ml of 0.9% NaCl aqueous solution (saline) intravenously. Rats in group B were the control group for group A, administered identical volume of saline solution intravenously. Rats in group C were the ADR + DMSO group. DMSO (4 g/kg b.wt.) was given intraperitoneally following the administration of ADR, and then an intraperitoneal injection of DMSO (4 g/kg b.wt.) was given daily until the 6th day. Rats in group D were the ADR + DMTU group. DMTU (500 mg/kg b.wt.) was given intraperitoneally 6 h before the ADR administration followed by the intraperitoneal injection of 250 mg/kg b.wt. daily until the 6th day. Rats in group E were the saline + DMSO group and rats in group F were the saline + DMTU group. Rats in group E and F were injected DMSO and DMTU by the procedure described above after the saline administration. Both the method of administration and the doses of DMSO and DMTU were based on those of previous studies [6,9]. Urine was collected over 24 h, and total protein excretion was determined by the pyrogallol sulfonaphthalein method on the 7th, 14th, 21st, and 28th day after the ADR or saline administration.

In vivo EPR measurement. A 700 MHz RF EPR spectrometer for in vivo study was constructed in our laboratory. This has already been described in previous reports [10–13]. It consisted of an EPR resonator, a main electromagnet, a pair of field scan coils, a pair of field modulation coils, and RF circuits for homodyne detection. The surface-coil-type resonator (SCR) [14] which consisted of a single-turn coil (10 mm inner diameter) and transmission lines (flexible coaxial cables with 50 Ω characteristic impedance) was used as an EPR resonator. This resonator can be positioned in any of several possible sites in living animals.

Ten rats in groups A, B, five rats in groups C, D, E, and F on the 7th day after ADR and saline administration were anesthetized with intraperitoneal administration of 50 mg/kg b.wt. of sodium pentobarbital after 1 night of starvation. Under anesthesia the right kidney of the rats was exposed by an incision from the back and each rat was restrained in a static magnetic field. A single-turn coil of the SCR was placed on the kidney. The TEMPOL solution that had been dissolved in saline was injected via the tail vein at a dose of 57 mg/kg b.wt. The EPR measurements were repeated every 4 s from 20 to 52 s after the injection of TEMPOL. Each spectrum was obtained from an average of 3 accumulations of 1 s scans. The spectroscopy settings were as follows: RF power, 52 mW at 710 MHz; static magnetic field, 25 mT; field modulation width, 0.2 mT at 100 kHz; scan speed, 10 mT/s; scan width, 10 mT; and time constant, 1 ms.

Microscopic localization of ADR in the glomerulus. Since ADR has special feature of fluorescence, we have used fluorescence microscopy for localization of ADR in the glomerulus following a single intravenous administration of the drug. The fluorescence at an excitation wavelength from 470 to 490 nm was detected using a high pressure mercury lamp, an ND filter set (IX-FLA, Olympus, Tokyo, Japan), and an emission wavelength above 515–550 nm. This was designed to detect fine fluorescence from the connected microscope (IX70, Olympus, Tokyo, Japan). In the initial studies, glomeruli of male Wistar rats were collected by a sieving method on the first day after the intravenous ADR and saline administration, on the 7th day and 14th day after the intravenous ADR administration. In the next studies, glomeruli obtained from the sieving method were used after the intravenous ADR administration and following DMSO or DMTU injection intraperitoneally for 7 days.

Statistical analysis. Statistical significance was estimated by Student's t test, and $p < 0.05$ was considered significant.

Results and discussion

Urinary protein due to oxidative stress

The 24 h excretion of urinary protein in the rats is shown in Fig. 1a. In the ADR group (A), urinary protein gradually increased and remarkable change was detectable from the 14th day compared to the control group (B). On the other hand, rats treated with DMSO, DMTU (C, D, respectively) had significantly lowered protein excretion as compared to the ADR group (A) on the 28th day, although the urinary protein in group C, D was higher than that in the control group B. Much of the tissue damage done by superoxide and hydrogen peroxide appears to arise by their metal-ion-dependent conversion into a highly reactive oxygen radical which is probably the hydroxyl radical. Direct detection of the hydroxyl radical in vivo is extremely difficult because of its high reactivity, so attempts to gain evidence for a role of this radical as a toxic agent in animal studies have involved the use of the hydroxyl radical scavenger. DMSO and DMTU are such scavengers and have been used to test the involvement of the hydroxyl radical in several animal models. Inhibition of the damage by the administration of these drugs has been taken as evidence that the hydroxyl radical is responsible for the damage.

Impaired RRA due to oxidative stress

The peak-to-peak height of the lowest component of the triplet spectra was defined as signal intensity (Fig. 1b). Good linearity on the semilogarithmic plots between signal intensity and time course after injection of TEMPOL was observed (the absolute value of the correlation coefficient was >0.99) which means that signal intensity decays exponentially and it follows first-order kinetics (Fig. 1c). $\ln 2/k$ shows the half-life, and k is the constant of first-order. The half-lives of TEMPOL on the 7th day after the ADR administration in the A–F group are shown in Fig. 1d. The half-lives of TEMPOL in group A were statistically longer than those in groups B, C, and D. The half-lives of TEMPOL in groups E and F were not shorter than those in group B. The hydroxyl radical is not visualized directly in vivo even

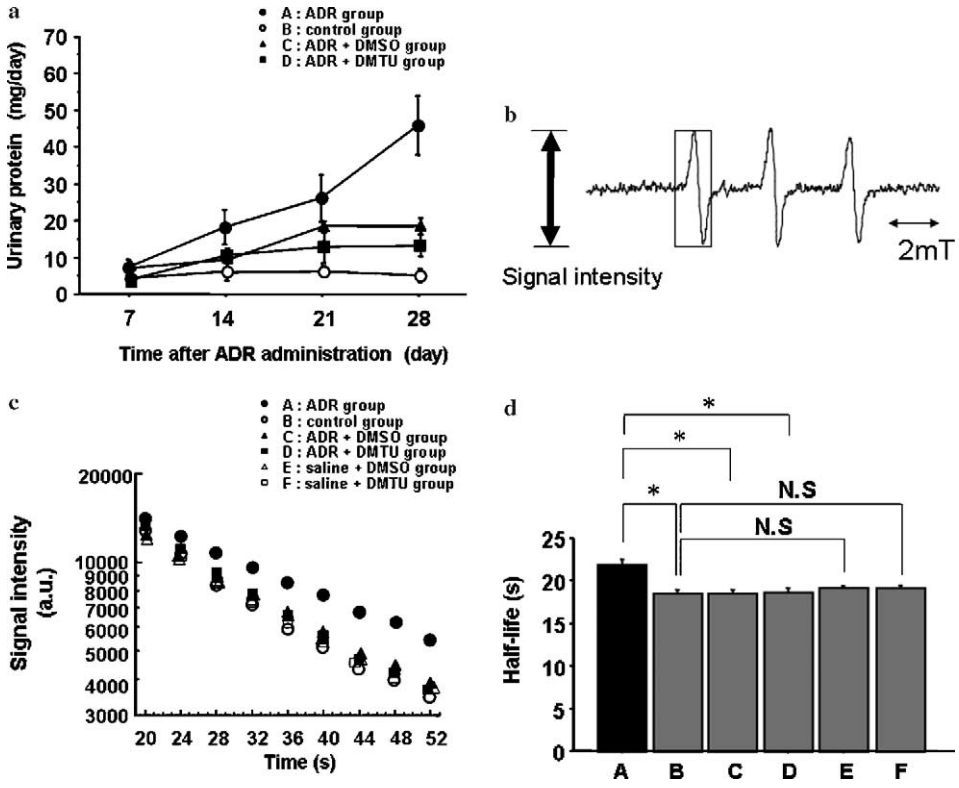

Fig. 1. (a) Time course of urinary protein in the ADR group (A), in the control group (B), and in the ADR group treated with DMSO (C), DMTU (D). The amount of urinary protein in each B, C, and D group is significantly different from that in group A on the 28th. The data shown are mean ± SE. (b) This is typical EPR spectra of TEMPOL. The peak-to-peak height of the lowest component of the triplet spectra was defined as signal intensity. (c) Typical semilogarithmic plots of signal intensity against time after the injection of TEMPOL in a rat of the ADR (A), the control (B), ADR + DMSO (C), ADR + DMTU (D), saline + DMSO (E), and saline + DMTU (F) group. The decay rate of the EPR spectra in the ADR group was slower than that of other groups. (d) The half-life of TEMPOL of the kidney in A–F groups. Groups A, B consist of 10 rats each and groups C, D, E, and F consist of 5 rats each. Values are expressed as means ± SE. $*p < 0.05$.

by the EPR method. On the other hand, the decay rate of the in vivo EPR signal intensity with nitroxide radical is indicative of the reducing ability. Using this spin probing method, we confirmed the efficacy of the antioxidant drugs DMSO, DMTU by in vivo EPR. In the present study, it showed that the antioxidants DMSO and DMTU did not contribute to the augmentation of RRA in the control group while they prevented the decline in RRA in the ADR group. To the best of our knowledge, this is the first report to show by an in vivo EPR method the efficacy of the antioxidant drugs DMSO and DMTU to improve RRA. In the present experiment, our results suggest that the protective effect of DMSO and DMTU on the kidney is mediated by their scavenging the hydroxyl radical which is induced by ADR distributed in the kidney. Excess oxidants or impaired RRA due to the hydroxyl radical generation results in renal injury associated with massive urinary protein. In our previous study, it was found that the RRA declined on the 7th day after ADR administration and recovered on the 14th day [8]. The impairment of the RRA occurred before the appearance of continuous urinary protein. Therefore, it suggests that impaired RRA in ADR nephropathy is an indicator predictive of continuous urinary protein.

Microscopic localization of ADR in the glomerulus

We show the microscopic fluorescent test in Fig. 2. The images of glomeruli on the 1st day after the ADR administration show strong orange fluorescence which is specific to ADR. It shows that the fluorescence on the 7th and 14th day after ADR administration was decreased compared to that on the 1st day. On the other hand, the orange fluorescence seen in glomeruli that were treated with DMSO and DMTU for 7 days is of the same degree as those that were administered ADR alone on the 7th day. There is a similar report, indicating a decrease in ADR concentration in some organs using fluorescence microscopy and high pressure liquid chromatography [15]. The improvement of the RRA could be associated with the decrease in ROS generation induced by ADR administration but the glomerular fluorescence in the antioxidant group was similar to that of the ADR alone group on the 7th day. Improvement of RRA could be caused by the scavenging activity of the hydroxyl radical with DMSO and DMTU rather than the decrease of ADR concentration in the glomerulus.

In summary, ADR-induced impaired RRA was inhibited by treatment with an antioxidant DMSO or DMTU.

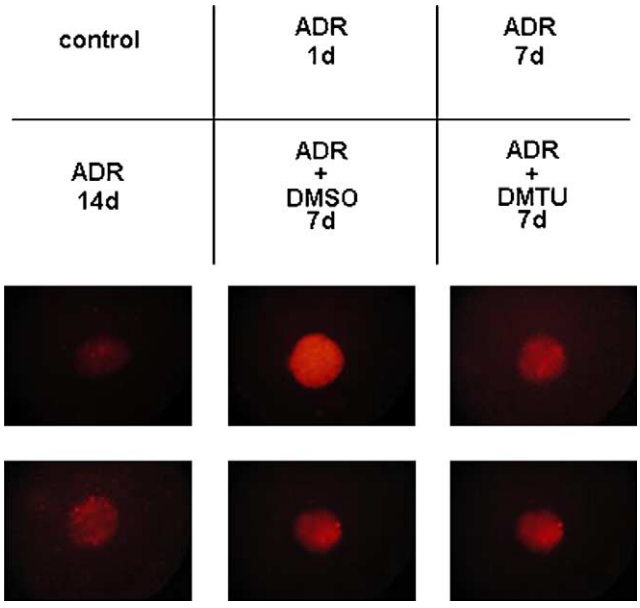

Fig. 2. The images of isolated glomeruli by fluorescence microscopy are shown in several groups, including the control, on the 1st, 7th, and 14th day after ADR administration and on the 7th day after ADR administration pretreated with DMSO or DMTU for 7 days.

The antioxidant ability of these drugs on the kidney was confirmed by the EPR method directly in vivo. Treatment with these drugs also suppressed ADR-induced urinary protein. These facts suggested that impaired RRA on the 7th day after the ADR administration was due to the oxidative stress which was a trigger of ADR-induced urinary protein.

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