Evaluation of adriamycin nephropathy by an in vivo electron paramagnetic resonance

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**Abstract**—A rat model for human minimal change nephropathy was obtained by the intravenous injection of adriamycin (ADR) at 5 mg/kg. By using an in vivo electron paramagnetic resonance (EPR) spectrometer operating at 700 MHz, the temporal changes in signal intensities of a nitrooxide radical, 4-hydroxyl 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), in the kidneys of rats with ADR nephropathy were investigated. The decay rate of the EPR signal intensity obtained in the kidney is indicative of the renal reducing ability. It was found that the reducing ability in the kidney declined on the 7th day after ADR administration and recovered after the 14th day. Impairment of the reducing ability occurred before the appearance of continuous urinary protein. The in vitro EPR study showed that this impairment of in vivo renal reducing ability is related to impairment of the reducing ability in the mitochondria.

**Key words**—Adriamycin nephropathy, Electron paramagnetic resonance, TEMPOL, Mitochondria, Reducing ability
Adriamycin (ADR)-induced nephropathy in rats is an experimental model for human minimal change nephropathy [1]. It is suggested that oxidative stress caused by reactive oxygen species (ROS) plays a role in its pathogenesis. In an in vitro experiment, it was reported that the hydroxyl radical was formed by a semiquinone radical which is generated from ADR in electron-transfer chains [2]. In an ex vivo experiment, Raats et al proved that ROS-mediated heparan sulfate depolymerization plays a role in ADR nephropathy and is correlated with glomerular basement membrane permeability [3]. Barbey et al [4] reported an increase in malondialdehyde (MDA) in the kidney about 1 week after ADR administration. Washio et al [5] and Okasora et al [6] indicated that scavengers for ROS showed nephroprotection against ADR-induced renal injury. 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 of the amount of oxidant and reductant. By using an EPR technique, in vivo reducing ability can be estimated [7]. The 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 [8]. However, nitroxide radicals are reduced to EPR silent compounds by various reactions because the reduction potential of nitroxide is relatively low [9]. The effect of reactions, other than reductions, on the metabolism of nitroxide is negligible. It is thought that the reduction sites are the respiratory chain in the mitochondria [10-12], microsomes [13-16] and several antioxidants in the cytosol [17-20].

We have already succeeded in measuring the temporal changes in EPR signal intensities in vivo in several organs, including the kidneys, of a living rat after the administration of a nitroxide radical, 4-hydroxyl-2,2,6,6-tetramethylpiperidin-1-oxyl (TEMPOL), and estimated the decay rate of TEMPOL in several organs. It is well known that exogenous nitroxide radicals are reduced in the liver and other organs and excreted via the kidney [15,21,22]. When nitroxide radical in the urine is not observed although it is distributed in the kidney, the decay rate of the renal TEMPOL signal reflects its reducing ability.

Using this method, we have already shown an impairment of in vivo reducing ability in the kidney on the 9th day after puromycin aminonucleoside (PAN) administration [23]. The relationship of this impairment and mitochondrial damage was also confirmed by an in vitro study. Each ADR-induced nephropathy and PAN-induced nephropathy shows nephrotic syndrome which is characterized by heavy proteinuria and hypoalbuminemia. It is thought that oxidative stress relates to the pathogenesis of those nephropathies. However, there were several differences between
ADR-induced nephropathy and PAN-induced nephropathy such as the time course of the appearance and the duration of urinary protein and the presumed site for impairment of protein synthesis. It is not clear whether ADR administration would decrease the in vivo reducing ability of the kidney.

In this study, the in vivo renal reducing ability of ADR-induced nephropathy in rats was investigated. Additional in vitro experiments were also undertaken to identify the mechanisms for change in reducing ability.

Material and methods

Chemicals

ADR, sucrose and mannitol were purchased from Wako Pure Chemical Industries (Osaka, Japan). TEMPOL, and superoxide dismutase (SOD) were obtained from Sigma Chemical Co. (St. Louis, MO). EDTA was purchased from Dojindo Laboratory (Kumamoto, Japan). All other chemicals and reagents were of analytical grade.

Animals and basic procedures

Male Wistar rats (180 – 200 g body wt) were given free access to standard laboratory chow and water. The rats were divided into ADR and control groups. The rats of the ADR group were administered 5 mg/kg b.wt of ADR dissolved in 1 ml of 0.9% NaCl aqueous solution (saline) intravenously and the control group received an identical volume of saline. Urine was collected over 24 h, and total protein excretion was determined by the pyrogallol sulfonphthalein method on 0, 7, 14, 21, and 28 d after 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 [7, 24-26]. 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) [27] which consisted of a single-turn coil (10 mm inner diameter) and transmission lines (flexible coaxial cables with 50 ohms 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 each group were anesthetized with intraperitoneal administration of 50 mg/kg b. wt. of sodium pentobarbital after 1 night 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 secs from 20 to 52 secs after the injection of TEMPOL. Each spectrum was obtained from an average of 3 accumulations of 1 sec 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.

The influence of some radical scavengers to the temporal changes in the EPR signal of TEMPOL was also tested. Ten rats of the ADR group, on the 21st day after ADR administration, were divided into two groups: SOD (n=5) and mannitol (n=5) groups. SOD at a dose of 52500 unit/kg b.wt or 4 ml of 20% mannitol/kg b.wt was used as a scavenger for the superoxide radical or the hydroxyl radical, respectively. Each scavenger was injected via the intravenous route 5 min before the TEMPOL injection. EPR measurements were performed by the procedure described above. The doses of SOD and mannitol were based on those of previous studies [3,28].

Preparation of homogenates and various fractions

The bilateral kidneys were removed under anesthesia on the 7th day after the ADR or saline injection. The samples on the 21st day after ADR administration were also collected. Each group consisted of six rats. The kidneys were immediately homogenized in 4 volumes of solution consisting of 0.25 M sucrose and 0.1 mM EDTA (pH 7.4) at 0°C using a Potter-Elvehjem Teflon homogenizer. This homogenate was defined as S0 and consisted of nuclear, mitochondrial, microsomal and cytosol fractions. Subcellular fractionation was achieved by differential centrifugation at 4°C. The nuclear fraction was excluded by centrifugation of the homogenate at 600 × g for 10 min and the supernatant from this step was defined as S1 which consisted of mitochondria, microsomes, and cytosol. Then the mitochondrial fraction was excluded by centrifugation of S1 at 8000 × g for 10 min and the supernatant from this step was defined as S2 which consisted of microsomes and cytosol. The microsomal fraction was excluded by centrifugation of S2 at 105,000 × g for 60 min and the supernatant was defined as S3. These samples from various fractions (S0 ~ S3) were deoxygenated by the bubbling of 100% nitrogen gas for 5 min at room temperature, and were mixed in an identical volume of a deoxygenated 0.25 M sucrose solution. The final concentration of the sample was 11% (w/v). The deoxygenated TEMPOL saline solution (1 mM) was added to the sample and was mixed quickly. Total volume of the reaction mixture was 500 μl consisting of 450 μl of the samples and 50 μl TEMPOL solutions.

In Vitro EPR measurement of homogenates

The in vitro EPR measurements were conducted using a conventional X-band EPR spectrometer (TE-200; JEOL, Tokyo, Japan). The samples containing the renal homogenate and
TEMPOL were put into capillary tubes (75 mm in length, 4 μl in inner volume). The capillary tube was inserted into an EPR quartz test tube (5 mm in outer diameter) which was set in the EPR cavity resonator. The EPR measurements were repeated every 20 secs from 2 to 7 min after the mixing of various fractions and TEMPOL solutions at 25°C. The spectroscopy settings were as follows; microwave power, 4 mW at 9.43 GHz; static magnetic field, 335 mT; field modulation width, 0.1 mT at 100 kHz; scan speed, 0.5 mT/s; scan width, 5 mT; and time constant, 1 ms.

In Vitro EPR measurement of urine

Rats on the 21st day after ADR or saline administration were used. Five min after the TEMPOL injection at a dose of 57 mg/kg via tail vein under anesthesia the bladder of the rat was exposed by an abdominal midline incision and urine was obtained by a syringe with needle. The urine was oxidized by an identical volume of 1 mM potassium ferricyanide and subjected to the X-band EPR spectrometer. The spectrometer settings were similar to those for the homogenates.

Tissue glutathione content

The renal total glutathione content was measured as an index of the tissue reductant level. The rats on the 0, 7, and 21st day after ADR administration were used. The kidneys were removed and 100 mg cortical tissue was homogenated in 1 ml of 5% sulfosalicylic acid. Centrifugation of the homogenate was conducted at 8000 × g for 10 min and the supernatant from this step was used for determination. The amount of tissue glutathione was determined using Total Glutathione Quantification Kit (Dojindo Molecular Technologies, Inc, Japan.).

Statistical analysis

Statistical significance was estimated by the Student's t-test, and \( p < .05 \) was considered significant.

Results and discussion

In vivo EPR measurement

The peak-to-peak height of the lowest component of the triplet spectra of TEMPOL defined the signal intensity. In all groups, good linearity on a semilogarithmic plot was observed (the absolute value of the correlation coefficient was >.99) which means that signal intensity decays exponentially. Typical plots are shown in Fig. 1-A. Because the temporal change of the signal intensity shows an exponential decay, its half-life can be used as an index of the decay rate. The half-lives in the ADR group and control group are shown in Fig. 1-B. Seven days after ADR
administration, the half-lives of TEMPO in the ADR group were statistically longer than those in the control group but this did not persist from 14 to 28 days. Continuous urinary protein appeared after this impairment in reducing ability (Fig. 1-C).

Because a single exponential decay of the EPR signal intensity was observed, the equation expressing the TEMPO concentration in a target organ (Xn) as a function of time (t) approximates

\[ X_n = X_{n0} \exp(-k_{0n}t) \]  

(1),

where \( X_{n0} \) is the initial value of the concentration and \( k_{0n} \), a first order rate constant (\( = \ln 2/\text{half-life} \)). However, the organs are actually connected to each other via the blood circulatory system. Thus, the temporal change in \( X_n \) can be expressed by

\[ \frac{dX_n}{dt} = k_{1n}X_v - k_{2n}X_n - k_{3n}X_n \]  

(2),

where \( k_{1n} \), \( k_{2n} \), and \( k_{3n} \) are the rate constant of the inflow, outflow, and reduction of TEMPO, respectively, in the target organ and \( X_v \) is the TEMPO concentration in the blood. By using data from a previous EPR kinetics study of multiple organs of rats [8, 30] \n
\[ X_n = C_{1n} \exp(-k_{3n}t) + C_{2n} \exp(-k_vt) \]  

(3)

can be obtained from Eq. (2), where \( C_{2n} = k_{1n}X_{v0}/(k_{3n} - k_v) \), \( C_{1n} = X_{n0} - C_{2n} \), \( k_v \) is the rate constant of the TEMPO concentration change in the blood, and \( X_{v0} \) the initial value of \( X_v \). \( C_{1n} \) and \( C_{2n} \) reflect the degree of influence of the target and other areas to the EPR signal decay observed in the target organ. The conditions under which the curve of Eq. (3) could fit the curve of Eq. (1) were simulated when the values obtained from the previous EPR measurements were substituted for \( k_{0n} \), \( k_v \), \( X_{n0} \), and \( X_{v0} \) in these equations [7, 29]. The results of simulation showed \( k_{0n} \sim k_{3n} \) and \( C_{1n} \gg C_{2n} \), indicating that \( X_n \) is not influenced by \( k_v \), and \( k_{0n} \) reflects \( k_{3n} \). Therefore we believe that the rate constant (i.e., half-life) of TEMPO measured in the kidney (i.e., the target organ) reflects the reduction of TEMPO in the area and is not influenced by the reduction in other areas when renal excretion can be neglected.

In vitro EPR measurement of homogenates

Because exponential decay of the TEMPO signal was also observed in the homogenates study, the half-life was used to estimate decay rates. The in vitro EPR study using various fractions of the kidney showed that the reducing ability in the homogenate and the supernatant (S1) of ADR-treated rats on the 7th day decreased remarkably compared to that in the controls (Fig.1-D). When the mitochondrial fraction was removed from the supernatant (S1) there is no significant difference in reducing ability between the two groups. These findings indicate that impairment of the in vivo renal reducing ability which was observed on the 7th day after ADR administration is related to an impairment of the reducing ability in the mitochondria. Muhammed et al showed that ADR inhibited oxidative phosphorylation in freshly prepared mitochondria from the heart, liver and
kidney of the rat [30]. In addition, we also reported mitochondrial impairment in PAN nephrosis[23].

**Change of the half-life of TEMPOL on the 21st day compared to that on the 7th day**

The in vivo renal reducing ability declined on the 7th day after ADR administration but was normalized on the 21st day. There are 4 plausible explanations for this normalization: (1) reaction between TEMPOL and other radicals, (2) excretion of TEMPOL, (3) hypoxia, and (4) augmentation of the reductant level on the 21st day after ADR administration. The nitroxide radical, including TEMPOL, has been reported to react with the hydroxyl radical and the superoxide anion radical in the presence of cysteine or NADH [31]. In their report, the EPR signal intensity of nitroxide radicals decays linearly in the reaction with other radicals. On the other hand, its EPR signal intensity decays exponentially in reactions with reductants. Therefore, the reaction dynamics of nitroxide radicals with reductants differs from that with other radicals. In the present study, the EPR signal intensity of TEMPOL decayed exponentially. Furthermore, the administration of radical scavenger (SOD and mannitol) did not influence the decay rate of TEMPOL in the kidney of the rat on the 21st day after ADR administration (Fig.2-A). Therefore, the reaction of TEMPOL with other radicals (such as the superoxide and the hydroxyl) was negligible. Concerning the normalization of reducing ability, we measured the urinary excretion of TEMPOL on the 21st day after ADR administration. No TEMPOL signal in the oxidized urine of the control rats was observed using an X-band EPR spectrometer 5 min after the tail vein TEMPOL injection. In the kidney, the decay rate of TEMPOL was influenced by renal reduction and excretion. The amount of TEMPOL excreted in the urine 5 min after TEMPOL injection corresponds to 1/500 of the amount of the administered TEMPOL on the 21st day after ADR administration. In this circumstance, the influence on the half-life by excretion was calculated to be less than 1%. Therefore, renal excretion in this study merely affects the renal half-life of TEMPOL. The rate of nitroxide reduction is enhanced under hypoxic conditions. From 7 to 21 days after ADR administration, urinary proteins increased gradually. This suggests that the animals have poor circulation leading to kidney hypoxia because of the decrease in plasma albumin concentration causing fluid shifts and a decline in plasma volume. Kuppusamy et al reported a hypoxic and highly reducing status in tumor tissues compared to those of normal tissues and showed an enhanced bioreduction of nitroxide using in vivo EPR spectroscopy [32]. The rate of nitroxide reduction under augmentation of reducing systems is enhanced. Glutathione is one such reductant. A previous report showed that the total glutathione content of renal tissue in ADR nephropathy tended to decrease on day 5, return to nearly normal level on day 7, then increase significantly on day 14 [33]. Furthermore, in general, the single administration of a glutathione-depleting drug is known to result in a transient decrease in glutathione level, followed by an increase to a level above
the normal range due to enhanced glutathione production [34]. This study proved that the amount of total glutathione on the 21st day after ADR administration was elevated compared to that on the 7th day (Fig.2-B). Glutathione does not reduce nitrooxide directly, but by enhancement of other reducing systems such as the ascorbic acid/dehydroascorbic acid system in which case the reduction of the nitrooxide radical was augmented [29]. Finally, we reached the conclusion that the reduction of the nitrooxide radical, augmented on the 21st day after ADR administration compared to that on the 7th day after ADR administration, was the cause of the elevated glutathione level in the present study. Moreover, it seems to call for further investigation concerning tissue oxygen concentrations in vivo.

In summary, it was found using in vivo EPR spectroscopy that the reducing ability in the kidney declined on the 7th day after ADR administration and recovered after the 14th day. Continuous proteinuria was not observed coincident with the impaired reducing ability but was preceeded by a decrease of reducing ability which was related to mitochondrial impairment.
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Legends

Fig. 1-A: Typical semilogarithmic plots of signal intensity against time after the injection of TEMPOL in a rat of the control (○) or ADR (●) group. Each rat received ADR or saline 7 days before the EPR study.

Fig. 1-B: The half-life of TEMPOL of the kidney in the control (○) and ADR (●) groups. Each group consists of 10 rats and values are expressed as mean ± SD. *p<.05.

Fig. 1-C: Time course of urinary protein in ADR nephropathy. The data shown are mean ± SD. The rats of the ADR group (●) were administered 5mg/kg body wt. of ADR dissolved in 1 ml of 0.9% NaCl solution (saline) in the tail vein while the control group (○) received an identical volume of saline. Urine was collected over 24 h, and total protein excretion was determined by the pyrogallol sulfonphthalein method. *p<.05.

Fig. 1-D: The half-life of TEMPOL in the reaction mixtures containing various fractions of the kidneys in the rats which received ADR on the 7th (black bar) and 21st days (gray bar) or saline on the 7th day (white bar). S0, homogenate; S1, supernatant without the nuclear fraction from S0; S2, supernatant without the mitochondrial fraction from S1; and S3, supernatant without the microsomal fraction from S2. Each group consists of six rats and values are expressed as mean ± SD. *p<.05.

Fig. 2-A: The half-life of the SOD- or mannitol-treated and the untreated rats on the 21st day after ADR administration. Each SOD- or mannitol-treated group consists of 5 rats and the untreated group consists of 10 rats.

Fig. 2-B: The renal total glutathione contents were measured on the 0, 7th, and 21st days after ADR administration. Each group consists of 6 rats and values are expressed as mean ± SD. *p<.05.
Fig. 2

A

Half-life (s)

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B

Total glutathione (μmol/g soft tissue)

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<th>Time after ADR administration (day)</th>
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<td>0</td>
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