In vivo imaging of oxidative stress in ischemia-reperfusion renal injury using electron paramagnetic resonance

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Oxidative stress is now known to play a key role in many diseases. As this role become clear, the importance of analyzing the kinetics of reactive oxygen species (ROS) or related substances in vivo will increase. Usually, oxidative stress-related reactions have very high rate constants. ROS very quickly react with intra- or extracellular neighboring substances, such as antioxidants, reducing molecules, lipids, and metals, and their half-lives are less than a millisecond. Therefore, oxidative stress-related reactions are often inevitably estimated indirectly by their end products or gene-related products. However, problems concerning the resemblance to and reproduction of in vivo circumstances are continuously open to criticism in these in vitro or ex vivo estimations, and there is a pressing need for the direct measurement of both oxidative stress and antioxidant status in vivo.

Electron paramagnetic resonance (EPR), which is a technique for the detection of unpaired electrons, has the potential to meet this need for direct measurement. Because of water-induced dielectric loss, the conventional X-band EPR system is not suitable for in vivo work. The recent development of low-frequency EPR has circumvented this problem, and in vivo EPR measurement is coming into use. In the condition of normal renal function, redox evaluation using the spin probe method and the usefulness of this technique are already established (5, 6, 11, 14, 27, 29, 35). We have previously reported EPR images representing renal and hepatic organ reducing activity in the Nrf2-deficient mouse (5), a newly developed oxidative, stress-related, lupus-like autoimmune disease model (34). In EPR measurements, organ reducing activity is often evaluated by the spin probe method. In this method, a paramagnetic stable spin probe (which is EPR positive in stable conditions) is injected into the animals. This spin probe is converted to the corresponding hydroxylamine by one-electron reduction and loses its EPR signal in organs. Consequently, the measurement of EPR signal intensity is equal to the measurement of organ reducing activity against radicals, which is a major factor of organ antioxidative activity (5, 14, 27). A group of intracellular molecules, including glutathione (GSH), NADPH, and ascorbates, contributes to maintain cellular reducing activity and redox status (21). Among them, GSH especially plays a key role in both spin probe reduction (11) and maintenance of the cellular redox environment (21). Nitrooxide spin probes do not chemically react with low-molecular-mass thiols directly; however, GSH is shown to possess a significant role in the bioreduction of nitroxides at the cellular or organellar level. A recent study using in vivo EPR imaging (EPR) showed a correlation between intracellular GSH level and reduction of 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-1-oxyl (carbamoyl-PROXYL) in radiation-induced fibrosarcoma tumor-bearing mice (11). On the other hand, changes in the half cell reduction potential of the GSSG/2GSH couple are shown to correlate with the cellular biological status in such areas as proliferation, differentiation, and apoptosis (21). Therefore, the measurement of spin probe reducing activity by EPR may provide various information about tissue radical-reducing activity, redox status, and biological status.

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Innovative Methodology

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EPR IMAGING OF ISCHEMIA-REPERFUSION RENAL FAILURE

The conventional in vivo EPR system detects the EPR signal from the whole of its resonator. Therefore, during its application to the upper abdominal area, both the liver and kidneys provoke an EPR signal, and it is difficult to determine the origin of the signal. In addition, water-soluble spin probes such as carbamoyl-PROXYL and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (tempol), which are commonly used in in vivo EPR studies, may be altered by the glomerular filtration rate (23). Therefore, to assess the kidney-reducing activity by EPR, we need to distinguish the reducing activity of the liver and kidneys and the signal decrease caused by renal reduction and excretion. To resolve these problems, we have developed EPRI to allow the measurement of EPR signal intensity in a selected area such as the kidney or liver. We applied this to the analysis of oxidative stress in ischemia-reperfusion acute renal failure (IR-ARF).

During the ischemia and reperfusion period, superoxide production in the kidney is markedly enhanced by the transformation of xanthine dehydrogenase to xanthine oxidase (18, 33) and the increase in free electrons in mitochondria, prosta-glandin H, and lipoxygenase with the coexistence of NAD(P)H (10) and infiltrated neutrophils (17). Superoxide raises the glandin H, and lipoxygenase with the coexistence of NAD(P)H (IR-ARF). Of oxidative stress in ischemia-reperfusion acute renal failure (IR-ARF).

Ischemia-reperfusion treatment. Female ICR mice, 8–10 wk old and weighing ~25 g, were used for the experiments. IR-ARF model mice underwent a left heminephrectomy and 30-min ischemia of the right kidney, followed by reperfusion. EPR, hematological, and biochemical measurements were performed before and 3 and 7 days after ischemia-reperfusion treatment. Mice that underwent skin incisions but had neither a nephrectomy nor ischemia treatment were designated as the control group. A group of mice that underwent a left heminephrectomy only was also employed for comparisons. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba.

Measurement of PC-OOH in organ homogenates. PC-OOH was measured by the method previously reported (4). In brief, the total lipids in each organ were extracted using chloroform and methanol. The total lipids were then separated by high-performance liquid chromatography using a methanol/water ratio of 95:5 (vol/vol) as the mobile phase. The PC-OOH peak was detected by an ultraviolet light detector at 235 nm and confirmed with a luminol and cytochrome c chemiluminescence method.

Quantitative measurement of reduced activity in the upper abdominal area using in vivo L-band EPR. L-band EPR measurements were conducted by the method previously reported, with minor modifications (5). The L-band in vivo EPR/EPRI system manufactured by JEOL (Tokyo, Japan), consisting of a 1-GHz microwave unit and a bridged four-gap loop-gap resonator (38-mm diameter and 28-mm length), was used in this study. Carbamoyl-PROXYL (300 mM, 2 ml/kg) was injected into the mice through the tail vein 15 min after pentobarbital sodium anesthesia. Each mouse was then placed in a plastic holder and put in the EPR system, with its upper abdominal area in the center and the bladder outside of the resonator. The signal intensity was measured using ESR-NT software (JEOL). Rate constants (k) were calculated from the EPR signal intensities measured every 20 s from 8 to 18 min after the carbamoyl-PROXYL injection. The peak-to-peak height of the lowest magnetic field signal in the triplet spectrum was defined as the signal intensity. EPR conditions for these in vivo measurements were magnetic field, 37.0 ± 5.0 mT; modulation width, 0.69 mT; time constant, 0.03 s; microwave power, 0.25 mW; and scanning time, 30 s. The carbamoyl-PROXYL signal intensity was semilogarithmically plotted against time, and the first-order spin reduction rate constant was estimated from the slope value of the observed clearance curve which was obtained by best fit. The half-life was calculated using the equation t½ = ln2/k.

Effect of superoxide-spin probe reaction on EPR measurement. To exclude the effect of a carbamoyl-PROXYL and ROS direct reaction on the signal intensities, changes in carbamoyl-PROXYL half-life by additions of a free radical-specific scavenger were measured. SOD (4 U/g) was injected intraperitoneally into the mice undergoing IR-ARF on preischemia and postischemia on days 3 and 7. Soon after the SOD injection, carbamoyl-PROXYL was injected and its half-life was measured using the method described above.

Semi-quantitative measurement of organ reducing activity using EPRI. 3D EPR images were obtained every 210–330 s until ~30 min after carbamoyl-PROXYL injection. 3D EPR images were constructed using ESR-CT version 1.183 software (JEOL). The EPR conditions were as follows: field gradient, 2.0 mT/cm; changing direction, 20° steps (provides 8 spectra for each projection); magnetic field, 37.0 ± 5.0 mT; microwave power, 0.25 mW; modulation width, 0.1 mT; time constant, 0.03 s; scanning time for each spectrum, 30 s; total scanning time, 210 s; and microwave frequency, ~1.100 MHz. A glass capillary tube 2 mm in diameter and 10 mm in length, filled with 12 mM carbamoyl-PROXYL, was attached to an area on the mouse corresponding to the removed left kidney and used as an internal standard.

On the obtained EPR image, a square area covering each organ was determined from the first image after carbamoyl-PROXYL injection. Different from MRI, the shape of each domain in EPRI is determined by signal intensity in the area rather than the organ outline. Therefore,
we needed to be careful to determine the area corresponding to each organ, and the area was confirmed not only with one cross-sectional image but with all 3D images (i.e., ZY, XY, and KZ planes). The signal intensity of the area was determined by the ratio of the signal value of the area and the internal standard (see Fig. 4B for a typical image). In the succeeding images, organ areas with exactly the same shape and size as that in the first image were determined, and the signal intensities from those areas were measured. Similar to the L-band measurement, the signal intensity was semilogarithmically plotted against time, and the approximate first-order spin reduction rate constant was estimated from the slope value of the observed clearance curve.

Ex vivo measurements of organ reducing activity. Ex vivo confirmation of reducing activity was measured by the method previously described, with minor modifications (5). In brief, mice were killed 7.5 min after carbamoyl-PROXYL injection, and the abdominal organs were removed. The remaining carbamoyl-PROXYL in the homogenates from these organs was measured by X-band EPR equipment (TX-25, JEOL) using a disposable flat EPR cell (Radical Research, Tokyo, Japan). The ratio of the intensity of the lowest magnetic field peak of carbamoyl-PROXYL to a manganese oxide internal standard peak was defined as the remaining carbamoyl-PROXYL signal intensity. After this measurement, the homogenates were combined with the same amount of 1.0 mM potassium ferricyanide. This process reoxidized EPR-silent hydroxylamines, which are already reduced by the tissue, to EPR-positive carbamoyl-PROXYL again. The signal intensity after the addition of potassium ferricyanide is defined as the total probe concentration distributed in the tissue (total probe) (24). The remaining carbamoyl-PROXYL/total probe ratio was used as an index for the tissue reducing activity.

Reagents. Carbamoyl-PROXYL was purchased from Aldrich Chemical (Milwaukee, WI). Potassium ferricyanide was obtained from Wako Chemical (Tokyo, Japan). All other chemicals were obtained from Sigma (St. Louis, MO) through Sigma Japan (Tokyo, Japan). All reagents were of the highest purity commercially available.

Statistical analysis. Multiple data comparisons were performed using the repeated-measures ANOVA with the Tukey-Kramer test. P values <0.05 were considered statistically significant. Results are expressed as means ± SD.

RESULTS

Effects of ischemia-reperfusion injury on creatinine, BUN, and PC-OOH. Mice treated with 30-min ischemia and a following reperfusion showed ARF. On day 3 after ischemia-reperfusion, serum creatinine and BUN rose significantly from 0.33 ± 0.05 to 0.73 ± 0.25 mg/dl and from 19.7 ± 3.9 to 54.7 ± 21.5 mg/dl, respectively (Fig. 1). Serum creatinine and BUN values decreased to 0.40 ± 0.12 and 27.1 ± 5.4 mg/dl, respectively, on day 7. The body weight of the mice decreased on day 3 and recovered on day 7; however, this change was not significant (data not shown). Hematocrit values continuously decreased until day 7 (data not shown). These parameters showed no remarkable changes in the control group and non-significant minor changes in the heminephrectomy group.

Before ischemia, the PC-OOH in the kidney and liver were 0.9 ± 0.6 and 1.3 ± 0.5 nmol/g tissue, respectively. They significantly increased to 4.7 ± 2.4 and 4.2 ± 2.1 nmol/g tissue on day 3 (P < 0.05) and partially recovered to 2.9 ± 1.7 and 2.9 ± 1.5 nmol/g tissue, respectively, on day 7 (Fig. 1). The PC-OOH concentration in the liver and kidney of the control and heminephrectomy group showed slight but not significant changes.

Carbamoyl-PROXYL half-life in the upper abdominal area. Typical three-line carbamoyl-PROXYL EPR signals were observed soon after injection and continuously observed for up to ~40 min. Showing good agreement with previous observations (15, 31), the plots of signal intensities were fitted to straight lines on a semilogarithmic scale with all mice, indicating that carbamoyl-PROXYL reduction obeyed first-order kinetics during the time measured (typical signal decay curves are shown in Fig. 2A). The half-life of carbamoyl-PROXYL decay in the upper abdominal area was 13.3 ± 1.1 min before ischemia reperfusion and was significantly prolonged to 22.7 ± 5.5 min on day 3 (Fig. 2B). On day 7, the half-life of the probe recovered to 14.5 ± 2.8 min. The prolongation of the half-life
METHODS. The addition of SOD and the sham operation made no significant difference. Recovery of carbamoyl-PROXYL decay is significantly prolonged on day 3 after ischemia-reperfusion. The figure shows sequential data from 1 mouse.

Confirmation of organ reducing activity by ex vivo EPR. To address whether these images truly correspond to the liver and kidneys, we euthanized the animals and removed their abdominal organs. The remaining carbamoyl-PROXYL/totai probe ratio was then used as an index of tissue reducing activity. Similar to the changes in half-lives on EPR, this ex vivo study revealed higher remaining carbamoyl-PROXYL/totai probe ratios in the kidney on day 3 after ischemia-reperfusion and its partial recovery on day 7 (P < 0.05, Fig. 5). The increase in this ratio was less remarkable in the liver on day 3, and the recovery to the control level on day 7 was more notable.

DISCUSSION

The metabolic disappearance of EPR signals from carbamoyl-PROXYL is determined by one-electron reduction (5, 24, 27). Glutathione is known to play a central role in paramagnetic loss among the various intracellular reductants (11). In addition to glutathione, the ascorbate cytochrome P-450, cytochrome P-450 reductase, and the mitochondrial electron transport system are all involved in nitroxide radical reduction in vivo (8, 14, 28, 32). In addition, this paramagnetic loss of nitroxide radicals does not occur through reduction with blood components but rather follows their transport to various organs (27). Thus the altered tissue antioxidant activity in organs results in the prolongation of their EPR signal decay.

Using the L-band EPR system, changes in nitroxide radical half-life occur in several pathophysiological conditions, including ischemic hypoxia of the brain (12), puromycin aminonucleoside-induced nephrosis (29), hepatic damage induced by carbon tetrachloride (6), and so on and are reported to reflect their antioxidative status as well. One report employed L-band EPR in the upper abdominal area of IR-ARF mice and found a decrease in the carbamoyl-PROXYL rate constant and its relationship to the ratio of reduced and oxidized glutathione (9). Until now, these quantitative L-band analyses have mainly been made in the whole area covered by the resonator, which means the total upper abdomen or head. Quantitative analysis in a specific organ or localized area has been examined in a few reports, such as the rate constant measurement in the murine brain and abdomen (7), hippocampus and cerebral cortex of rats after epileptic seizures (35), or in the tumor area of radiation-induced fibrosarcoma-bearing mice (11). The kinetics of carbamoyl-PROXYL are also affected by its removal to the area outside the resonator. Carbamoyl-PROXYL is water soluble and is transported to the liver and bilateral kidneys is imaged (Fig. 3). The esti-
soluble (23), and its transfer to the outside of the resonator is equal to excretion in the urine in our measurements. On the other hand, the kidney has the largest reducing activity for nitroxide radicals per unit mass among the internal organs (30). Therefore, to assess the renal reducing activity by EPR, we need to distinguish the effect by decreased glomerular filtration rate (GFR) and renal reducing activity. In our result, the L-band EPR measurement of reducing activity in the upper abdominal area and EPRI-measured reducing activity in the kidney is different on day 7. The results from ex vivo measurement with organ homogenates and from PC-OOH measurement agreed with that of EPRI. The change in L-band EPR-measured carbamoyl-PROXYL half-lives resembles that of creatinine, BUN, and EPRI-measured hepatic reducing activity. In addition, the liver is also known to be oxidatively damaged in the renal ischemia-reperfusion process (22). These results suggest that the L-band EPR measurement of carbamoyl-PROXYL on the whole upper abdominal area is affected by GFR and hepatic reducing activity and does not reflect renal reducing activity in renal insufficiency. In other words, EPRI is a suitable method for the noninvasive evaluation of oxidative stress especially in the presence of renal injury.

Some nitroxide radicals are considered to react with hydroxyl radicals or other ROS and lose their paramagnetic properties, which consequently leads to an accelerated EPR signal disappearance (3, 14, 19). Therefore, the balance between the reducing activity of tissue and local free radical reactions determines the signal decay rate of carbamoyl-PROXYL. Our results demonstrate a significant delay in the EPR signal decay of carbamoyl-PROXYL in the kidneys of IR-ARF mice. Although there remains the possibility that

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**Fig. 3.** EPR images of transaxial sections of the upper abdominal area. A typical series of three-dimensional EPR images of the control mouse are shown at 10 (A) and 14 min (B) after carbamoyl-PROXYL injection. High signal intensity is indicated in red and low intensity in blue. In A and B, the tiled image in the top left corner was taken in the most cephalic section, and the image in the bottom right corner was taken in the most caudal section of the abdomen. The thickness of each slice was ~0.3 mm. Close-up views of image 61 in A and B are shown in C and D, respectively. The high-intensity area in the center of each tiled image represents the liver. The high-intensity area adjoining the liver on the left abdominal side represents the left kidney, and the area on the right side of the liver represents the right kidney. The liver is 15 mm in axial length and 13 mm in diameter, and the kidney is 6 mm in axial length and 4 mm in diameter, estimated in A and C. The images of the kidneys disappeared, and the liver image was diminished in B and D. Lt., left; Rt., right.
superoxide generated in the organs of IR-ARF mice may affect carbamoyl-PROXYL half-lives, our experiment using SOD showed no such change. The in vivo half-life of SOD is short. However, the method we employed was sufficient to decrease carbamoyl-PROXYL half-life in a condition in which superoxide production is dominant (3, 20). Therefore, in our results, the direct reaction between carbamoyl-PROXYL and superoxide is less prominent and the change in carbamoyl-PROXYL half-life purely reflects reducing activity.

Until now, it has been difficult to describe renal reducing activity, especially for free radicals in vivo. Our study shows the usefulness of EPRI in measuring renal reducing activity and the evaluation of oxidative stress in renal disease, particularly involving renal dysfunction. Since the invasiveness of EPRI is quite low, this method can be relevant to several aspects of pathophysiological or pharmacological investigation. The evaluation of the antioxidative or redox-related effects of drugs might be a promising application.

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