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In vivo detection of intrinsic reactive oxygen species using acyl-protected hydroxylamine in puromycin nephrosis

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Abstract
Intrinsic reactive oxygen species (ROS) in a rat model of human minimal change nephropathy were detected directly using an *in vivo* electron paramagnetic resonance (EPR) method with 1-acetoxy-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine (ACP) in real time. The nephrosis was induced by the intravenous administration of 75 mg/kg of puromycin aminonucleoside (PAN). It was found that ROS in the kidney were increased 1 h after the administration of PAN. This increased oxidative stress declined at 24 h and returned to a normal level 3 days after PAN administration. This is the first non-invasive *in vivo* detection and quantification of specific ROS in an experimental nephrosis model.

Keywords: *Reactive oxygen species, acyl-protected hydroxylamine, puromycin aminonucleoside nephrosis, electron paramagnetic resonance*

Introduction

A number of studies have implied that reactive oxygen species (ROS) play a role in the pathogenesis of puromycin aminonucleoside (PAN) nephrosis in rats, a model of human minimal change nephropathy. Several *in vitro* and *ex vivo* studies have been conducted to examine the generation of ROS or antioxidant activity in PAN nephrosis [1,2]. Other reports demonstrated that free radical scavengers and other substances attenuate PAN-induced proteinuria [3–6]. These findings suggest that free radicals play an important role in PAN nephrosis. However, it is difficult to determine the internal ROS in PAN nephrosis *in vivo* directly.

Electron paramagnetic resonance (EPR) spectroscopy is a unique technique for the direct detection of electron spins, and enables the measurement of free radicals in a living animal in real time. We have already succeeded in measuring the temporal change in EPR signal intensities in the PAN-induced nephrotic rat's kidney after the administration of a nitroxide radical. The decay rate of the administered nitroxide radical reflects the

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biological reducing activity [7]. This spin probing method is useful for evaluating the redox status in the target organ *in vivo*. Regardless of these developments, the internal ROS have rarely been determined *in vivo* directly in disease models because of their short life and presence in small quantities [8], and the ROS were mostly detected in *in vitro* and *ex vivo* studies [9–11].

An acyl-protected hydroxylamine, 1-acetoxy-3-carbamoyl-2,2,5,5-tetramethylpyrrolidine (ACP, Figure 1A), has been developed as a new spin reagent for EPR measurements of intracellular oxidative stress [12]. This spin reagent is a stable non-radical compound that is not affected by oxidation outside of cells because of acyl-protection. However, inside cells, ACP is easily deprotected with intracellular esterase to yield hydroxylamine, which is oxidized to an EPR-detectable stable nitroxide radical by intracellular ROS. On the basis of an EPR signal intensity of this radical, intracellular oxidative stress can be estimated. To evaluate the oxidative activity in the target organ *in vivo*, we applied this new spin reagent to a rat model of PAN nephrosis. Firstly, we quantified the amount of specific ROS in kidneys under normal condition *in vivo*. Then, we verified the oxidative temporal change in an initial phase of the PAN nephrosis. Using several radical scavengers we determined which ROS were generated in this phase and roughly estimated the amounts of these radicals.

Materials and methods

Chemicals

ACP was synthesized in our laboratory as previously described [12]. PAN, superoxide dismutase (SOD), catalase and 4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries (Osaka, Japan). All chemicals and reagents were of analytical grade.

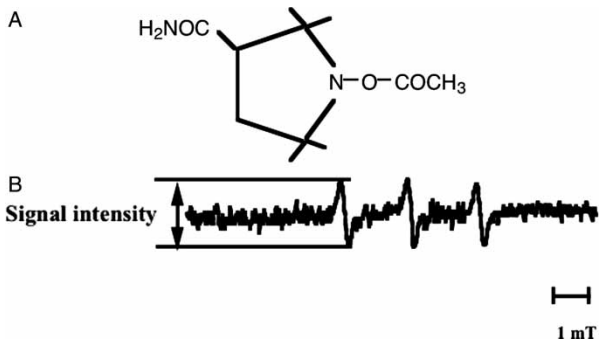

Figure 1. Chemical structure of ACP (A) and typical EPR signal after the injection of ACP (B). Signal intensity was the peak height of the lowest component of the EPR triplet spectra.

Animals and induction of PAN nephrosis

Male Wistar rats (250–300 g b.wt.) were given free access to standard laboratory chow and water, and then used for experiments after one night of starvation. The rats were divided into two groups, the PAN nephrosis and the controls. The rats of the PAN nephrosis group were administered 75 mg/kg b.wt. of PAN dissolved in 1 ml of 0.9% NaCl (saline) via the tail vein to induce nephrosis. Five rats were used for EPR measurements at 1, 4, 12, 24, 72 and 120 h after PAN injection. Different rats were used for EPR measurements at each time points. The control groups (5 rats) were measured immediately and at 1, 24 and 120 h after same volume of saline injection (expressed as group C in Figure 2). All procedures on animals were conducted in accord with National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Measurements of PAN induced oxidative stress using ACP

A 700 MHz RF EPR spectrometer for *in vivo* study was constructed at our laboratory. It consisted of a surface-coil-type resonator (SCR) resonator, a main electromagnet, a pair of field scan coils, a pair of field modulation coils, power supplies, a personal computer, and RF circuit for homodyne detection. The SCR was constituted from a single-turn coil (10 mm in inner diameter) and transmission lines (flexible coaxial cables with 50 ohms characteristic impedance). The SCR was connected to the microwave circuit through a three-stub tuner and driven at a frequency of approximately 720 MHz. This resonator can be positioned in any of several possible sites in living animals. More details of the

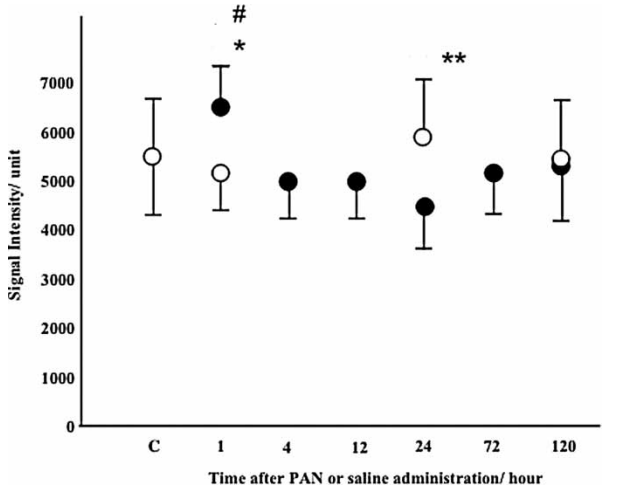

Figure 2. Average values of signal intensity after the injection of PAN (black circle) and saline (white circle). Each group consists of five different rats. Group C: immediately after the injection of saline *; $p < 0.001$, vs. group C, **; $p < 0.0001$, vs. group C, #; $p < 0.05$, vs. saline injected group.

system and SCR have already been described in previous reports [13–16].

Rats were anesthetized via intraperitoneal administration of 50 mg/kg b.wt. of sodium pentobarbital. Under anesthesia, the left kidney of the rat was exposed by an incision from the back and each animal was restrained in the static magnetic field. Thereafter an SCR was attached to the kidney. The rats in each group received via the tail vein an ACP solution (0.4 mmol/kg b.wt.) that had been prepared by dissolving in saline. All the rats (PAN and controls) were treated with ACP. The EPR measurements were repeated every 2 min from 5 to 15 min after the injection of ACP. One spectrum was obtained from an average of 32 accumulations of 1 s scan. The peak height of the component of the triplet spectra appeared in the lowest magnetic field and was defined as the signal intensity (Figure 1B). The average values of the signal intensities for the measuring period were calculated. The spectroscopy settings were as follows: RF power, 52 mW at 720 MHz; static magnetic field, 26 mT; field modulation width, 0.2 mT at 100 kHz; scan speed, 10 mT/s; scan width, 10 mT; time constant, 1 ms.

The concentrations of the nitroxide radical oxidized from ACP was roughly estimated by the contrast to the signal peak of the phantom containing 1 mM of 4-hydroxy 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL), attached to the SCR. The phantom is a cylindrical agar medium (10 mm in diameter, 15 mm axial length). This signal intensity was obtained over one spectrum. EPR conditions were the same as those already described.

Determination of increased ROS in PAN nephrosis

This experiment was performed to determine the kind of increased radical species in a rat's kidney 1 h after the injection of PAN. Rats were divided into the control group which received saline and the PAN group which received 75 mg/kg b.wt. of PAN intravenously. SOD (9,000 units), DMSO (200 mg) or catalase (112,000 units) was dissolved in 1 ml of saline. Both group of rats received either 0.5 ml of SOD, DMSO, catalase or saline 30 min prior to and 30 min after saline or PAN administration. After the injection of ACP, EPR measurements were performed in the same manner as the previous experiment.

Statistical analysis

Statistical analysis was performed using Statview 5.1 computer software (Abacus Concepts Incorporation, USA). Data are presented as the mean ± SD and analyzed by factorial analysis of valiance (ANOVA) and *post hoc* comparisons. One data (Figure 2) are analyzed by the Student's *t*-test, and *p* values less than 0.05 were considered significant.

Results

Measurement of EPR signals from ACP

ACP injected in rats showed triplet an EPR signal which agrees with the spectra of 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine, a deacylated form of ACP. EPR signal intensities increased after the injection of ACP. They reached the maximum level at 5 min and remained at the same level during the measuring period. Figure 1B shows a typical EPR spectrum from the kidney 5 min after the injection of ACP in the control group (group C). The signal intensity of a phantom containing a 1 mM TEMPOL solution was set at 100,000 units. That means 1 unit of signal intensity obtained from the data can be roughly estimated to be about 0.01 μM of nitroxide radical concentration.

Time course of oxidative stress in PAN nephrosis

The average values of the EPR signal intensities repeatedly measured every 2 min from 5 to 15 min after ACP injection (Figure 2). EPR signal was also observed in the saline injected rats (control group). The signal intensities measured at 1, 24 and 120 h after saline injections were 5021 ± 771, 5858 ± 1259 and 5480 ± 1393, respectively, and there were not statistically significant from each other. The EPR signal intensity at 1 h after PAN administration was 6290 ± 930 and this was significantly higher than that of the control group. Twenty-four hours after PAN administration, the EPR signal intensity was decreased to 4421 ± 1195, and this was significantly smaller than that of the control. After that time, the signal intensities were gradually increased and there were no statistical differences.

Determination of increased ROS in PAN nephrosis

The average values of signal intensities after the injection of ACP with SOD, DMSO or catalase 1 h after the administration of saline and PAN are shown for controls in Figure 3 and for PAN nephrosis in Figure 4. In the control group, the values in the groups treated with SOD (4310 ± 860) or catalase (4500 ± 980) were decreased compared to the single saline treated group (5510 ± 1350). However, DMSO showed no effect (5040 ± 1180). In the PAN group, the values in the groups treated with SOD (4370 ± 1220), DMSO (4480 ± 870) and catalase (4400 ± 410) declined remarkably compared to the single PAN treated group (6290 ± 930).

Discussion

An induction of ROS by PAN is generally accepted as shown in previous reports with isolated glomeruli [2,17] or kidney-slices [1]. The acute phase ROS

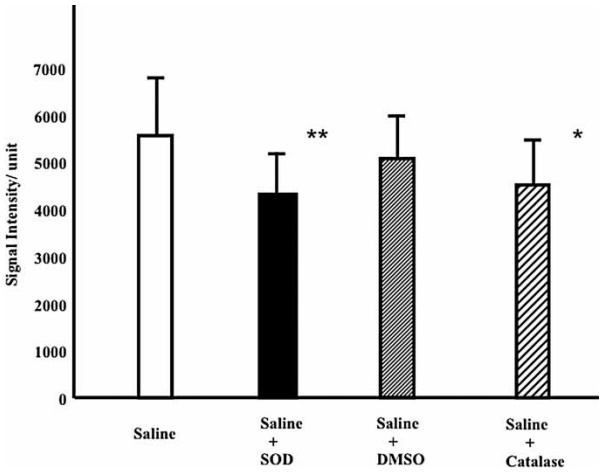

Figure 3. Average values of signal intensity after the injection of ACP with SOD, DMSO or catalase in the control group. These rats were used at 1 h after saline injection and each group consists of five rats. *; $p < 0.001$, vs. saline, **; $p < 0.0001$, vs. saline.

induction occurs within 15–60 min after PAN injection [1,2,17]. A generation of hydroperoxides in isolated glomeruli from a nephritic rat 7 days after a PAN injection is also reported [17]. However, it was difficult to determine the *in vivo* ROS directly because of their short life span and presence in small quantities. *In vivo* studies for verifying the oxidative temporal change in an initial phase of PAN nephrosis have never been performed. In this study, we have succeeded in measuring the generated ROS from the rat's kidney in PAN nephrosis directly using an EPR method with ACP reagents in real time. Our *in vivo* results revealed that the increase of ROS occurs only 1 h after the PAN administration and is consistent with the previous *ex vivo* and *in vitro* experiments.

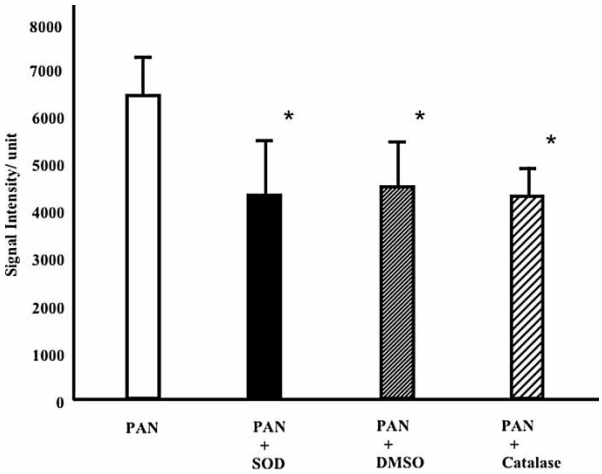

Figure 4. Average values of signal intensity after the injection of ACP with SOD, DMSO or catalase in PAN group. These rats were used at 1 h after PAN injection and each group consists of five rats *; $p < 0.0001$, vs. PAN.

Moreover, our result revealed that this increased ROS level was about 800 units of EPR signal intensity, which amount was equivalent to 8 μM of nitroxide radical concentration.

The signal intensities 24 h after PAN administration were significantly smaller than those of the controls. This phenomenon leads to speculation that an intrinsic antioxidant enzyme or substance was increased or induced as a biological antioxidant defense brought on by the drastic ROS generation induced by PAN and then, it suppressed the generation of ROS transiently at this point. However, changes in antioxidative enzymes such as SOD, reduced glutathione and catalase within 24 h after PAN administration have not been reported and further investigation is indicated.

Because the EPR signal intensity derived from acyl-protected hydroxylamine reflects nonspecific oxidation (i.e. oxidative stress in a broad sense) in the cell, we needed to determine the oxidants using their specific scavengers [12]. We subsequently show by *in vivo* experiment that DMSO, SOD and catalase inhibit the increases of ACP signal by PAN, indicating that ROS originated from or interacted with the hydroxyl radical, superoxide anion and hydrogen peroxide are increased in PAN nephrosis. Our experimental method allowed us to estimate a rough amount of ROS. Calculating from the decreased EPR signal intensities brought by SOD, DMSO and catalase, the total amount of the generated ROS with PAN treatment were equivalent to 20 μM of nitroxide radical. Quite interestingly, SOD and catalase decreased the EPR signal intensity after saline injection suggesting that ROS originated from or interacted with superoxide anion and hydrogen peroxide were generated in the kidney under physiological conditions. Other possibility is that these scavengers diminished single species which was considered as peroxynitrite. A recent report shows that peroxynitrite may react with cyclic hydroxylamines producing stable nitroxides [18]. DMSO and SOD inhibit this reaction by directly reacting with peroxynitrite [18] or superoxide, respectively. Another paper shows that the increased hydrogen peroxide formed by the addition of SOD will lead to increase in superoxide production and, finally, generation of peroxynitrite [19]. As these reports, peroxynitrite production may occur in the initial phase of PAN nephrosis, or may increase all those radicals such as hydroxyl radical, superoxide anion, hydrogen peroxide and peroxynitrite.

There are several reports examining the redox status in the kidney using an *in vivo* EPR technique. We evaluated the reducing activity of Nrf2-deficient mice [20] and succeeded in imaging the reducing activity of the kidney in ischemia-reperfusion acute renal failure mice [21]. In addition, we have already reported a decreased reducing activity in rat's kidneys from 7 to 14 days after PAN administration [7] and

also demonstrated an impaired reducing activity in kidneys after adriamycin administration [22]. In these studies, a combination of an *in vivo* EPR spectroscopy and a spin probing method enabled us to evaluate the redox status of the target organ in a living animal in real time. However, it was still difficult to detect the internally generated free radicals because administered exogenous nitroxide radicals are reduced to EPR silent compounds in the mitochondria [23,24], microsomes [25,26], and cytosol [27,28].

ACP is a stable non-radical compound but is easily converted to hydroxylamine by an intracellular esterase. Hydroxylamine, thus formed, is oxidized by reaction with ROS to yield an EPR-detectable nitroxide radical. Therefore, the EPR signal from ACP administered to a living animal reflects the strength of internally generated ROS quite directly. Yokoyama et al. applied this method to a kainic acid induced epileptic seizure model in rats and demonstrated that oxidative stress increased in the hippocampus and striatum in the rat brain [29]. The EPR signal intensity derived from ACP reflects non-specific oxidation in the cell. However, like in our present experiment, the administration of several radical-specific scavengers allows us to identify the generated ROS.

In summary, we showed that oxidative stress in the kidney was increased 1 h after the administration of PAN. These increased ROS were concerned with superoxide, hydroxyl radicals, hydrogen peroxide and/or peroxynitrite. However, the increased oxidative stress declined at 24 h and returned to normal levels. This is the first non-invasive *in vivo* detection and quantification of specific ROS in an experimental nephrosis model. Thus, ACP is a useful and powerful reagent to evaluate the oxidative status in various organs in living animals, and may serve effectively in studies of the pathophysiological role of ROS.

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