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Measurement of Oxidative Stress in the Rodent Brain Using Computerized Electron Spin Resonance Tomography

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The strategy of this study was to improve the electronic spin resonance (ESR) application used to detect free radical-induced oxidative stress in animal models. We have developed an *in vivo* ESR imaging system with high-quality ESR-computed tomography (CT) images by using a nitroxyl spin probe—BBB-permeable, 3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine-1-yloxy (MC-PROXYL)—in living small animals. We first measured the distribution of MC-PROXYL in the head region of a living mouse by using ESR-CT imaging after treatment with MC-PROXYL. In the ESR-CT experiments, it was clearly observed that MC-PROXYL was well distributed in the brain of head region of a living mouse. The ESR-CT images taken after treatment with MC-PROXYL demonstrate that the decay of MC-PROXYL in the isolated brain of a spontaneously hypertensive rat (SHR) was more rapid than that in a Wister Kyoto rat. ESR-CT analysis revealed that the region of rapid decay might be located in the cerebral cortex of the isolated brain of an SHR. These results suggest that the present study of ESR-CT imaging would be a useful tool for monitoring and detecting the locations of oxidative stress in the brains of rodent animal models.

Keywords: *nitroxyl spin probe, ESR-CT imaging, oxidative stress, brain, spontaneously hypertensive rat*

Introduction

In recent years, free radicals have been implicated in the pathogenesis of a wide variety of disorders. The ESR technique is specific only to free radicals and provides direct evidence of their presence. We have sought to develop a biomedical application of the ESR spin trap technique for the detection of free radicals.^{1–3} Electron spin resonance (ESR) imaging techniques have made possible the spatial mapping of unpaired electrons of free radicals in isolated organs and other *ex vivo* and *in vivo* biological systems.^{4–6} It has been reported that Kuppusamy et al.^{5,7,8} have demonstrated various biomedical applications of ESR imaging to biological organs.

A stable nitroxyl radical such as 3-carbamoyl-2,2,5,5-tetramethyl-pyrrolidine-1-yloxy (carbamoyl-PROXYL) or 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-yloxy (hydroxy-TEMPO) is often used as a probe for *in vivo* ESR operating in the lower frequency microwave bands (up to 1 GHz), where the dielectric losses of biological systems are lower. The nitroxyl spin probe reportedly loses its paramagnetism when exposed to a reducing agent in biological systems.^{9–11} Changes in the physiological redox state in response to oxidative stress caused by aging, ischemia-reperfusion, hyperoxia and hypoxia impact the decay rates of the nitroxyl spin probe.^{12,13}

The brain might be especially susceptible to oxidative stress, and it is well known that oxidative stress is associated with various brain diseases.¹⁴ Therefore, oxidative damage in the brain is currently a very active area of investigation. With the use

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of a suitable nitroxyl spin probe, which can pass through the blood-brain-barrier (BBB), the redox reaction in the brain could be monitored in living small animals through *in vivo* ESR imaging techniques. It has been reported that 3-methoxycarbonyl-2,2,5,5-tetramethyl-pyrrolidine-1-yloxy (MC-PROXYL) is a useful spin probe for the study of free radical reactions in the brain by means of ESR imaging.¹⁵⁻¹⁹ In the present study, we succeed in developing an *in vivo* ESR-computed tomography (CT) system capable of obtaining 3D distribution images of a BBB-permeable nitroxyl spin probe, MC-PROXYL. This technique was applied to the isolated brain of an animal model of essential hypertension, the spontaneously hypertensive rat (SHR), which has several characteristics of increased oxidative stress,^{20,21} in contrast to the Wister Kyoto rat (WKY).

Materials and Methods

Spin probe

MC-PROXYL was synthesized from 3-carboxy-2,2,5,5-tetramethyl-pyrrolidine-1-yloxy (carboxy-PROXYL; Tokyo Kasei, Tokyo, Japan) and diazomethane (Wako Chemical, Osaka, Japan) by the method described previously.^{22,23}

An ESR imaging system constructed in our laboratory and the JEOL ESR application laboratory was used. This system comprised a commercially available electromagnet (modified RE3X, JEOL, Tokyo, Japan), a pair of field scan coils, power supplies, a PC based on a Hewlett-Packard (KAYAK XW/400) computer using laboratory-developed software, and a 1 GHz microwave unit containing a four-window loop-gap resonator. The system is provided with four different coil sets, three for the gradients and one for rapid scanning. A setting of 0.9 mT/cm produced a field gradient of maximum strength. The gradient field was controlled with a current stabilizer, which was controlled by the PC mentioned above. The ESR-CT images were constructed according to Lauterbur's method,²⁴ known as 3D zeugmatography.²⁵ We applied linear magnetic field gradients along the *x*, *y*, and *z* axes produced by the magnetic field gradient coils. For 2D imaging, 36 projections alternating between gradient and nongradient were acquired in 55 s. Each projection required 1,024 points of data acquisition for imaging. The ESR absorption spectra were obtained by integrating the derivative spectrum with the recorded gradient. The mid-field hyperfine line in the spectrum was separated from the triplet signal of the nitroxyl radicals. Each signal data set was convoluted with

Shepp's filter function into the Fourier domain before the inverse Fourier transformation was performed on the spatial domain.²⁵ The 2D imaging pictures of 512 × 512 pixels were obtained from 18 projections for a gradient step of 10° in the spatial domain. For the ESR-CT imaging, the scanning plane was assigned an arbitrary set of two axes among *x*, *y* and *z*. Rotation of the plane was controlled by the remaining axis. The ESR-CT images were reconstructed from 18 planes of 2D imaging in different directions. Total acquisition time for this set of 324 projections (18 × 18) was 12 min for 128 slice pictures of 3D imaging. The distribution of a cubic matrix of 128 × 128 × 128 pixels was reconstructed with computer software (IRIS Explorer, Numerical Algorithms Group Ltd., Oxford, UK) through interpolation of the spin probe intensity. Instrument settings for ESR detection of MC-PROXYL were as follows: microwave power: 1 mW, magnetic field: 31.0–34.0 ± 5 mT, field modulation width: 0.1 mT, receiver gain: 63–100, time constant: 0.03 s.

Animal preparation for the ESR studies

ICR mice were used for the *in vivo* ESR imaging. The mice were anesthetized with 65 mg/kg pentobarbital (Dinabot, Osaka, Japan) by intraperitoneal injection. With this dose, anesthesia could be maintained for a period of up to 2.5 hours. The respiration rate was also monitored in selected mice by observing changes in the microwave reflections. After treatment with the nitroxyl compounds tested (140 mM, 10 ml/kg) via the tail vein, each animal was automatically restrained in the ESR unit, with its interaural line (vertical zero plane) aligned with the center of the resonator.

The WKY and SHR were used for ESR imaging of the isolated rat brain. The rats were anesthetized with 40 mg/kg pentobarbital by intraperitoneal injection, which was followed by 10 mg/kg of 140 mM MC-PROXYL solution via the tail vein. The brain was isolated 30 s after the treatment. In most experiments, the brain was restrained in a four-window loop-gap resonator just after the isolation, with the bregma aligned with the center of the resonator. ESR measurement was subsequently performed.

Results

We non-invasively demonstrated the data of the slice pattern of the ESR-CT images (in the *x-y* plane) of MC-PROXYL distribution in the head regions of living mice (Fig. 1). Figure 1 illustrates that the images of MC-PROXYL were used as gray-scale pixels. The insert figures (Fig. 1B) are

Fig. 1. A typical slice pattern of 3D ESR-CT images (in the x - y plane) obtained three minutes after the treatment with MC-PROXYL in the head region of a living mouse. To assign the anatomic position, representative ESR-CT images in slices in the x - y plane (0, the midsagittal section) derived from the corresponding images in panel B are a picture of superimposed by mouse studied. The ESR-CT images of MC-PROXYL were used as gray-scale pixels. Experimental conditions were as described under “Materials and Methods.”

superimposed with a picture of the mouse studied, which shows a clear distribution in the region corresponding to the brain. From these ESR-CT images, we demonstrated that MC-PROXYL was thoroughly distributed in the brain. Similar images were obtained in repeated imaging experiments ($n = 4$) on the distribution in the mouse head region. These results are highly consistent with the distribution of MC-PROXYL in the isolated rat brain as revealed with the ESR imaging system, reported previously.¹⁶⁻¹⁹

We confirmed that MC-PROXYL is also a suitable spin probe for the study of free radical reactions in the brain of the rodent animal model with ESR-CT imaging. In our recent study,¹⁹ these methods were also applied to the measurement of the oxidative stress on isolated brains from stroke-prone SHR (SHRSP) by means of ESR 2D imaging. We have demonstrated that oxidative stress, which has been reported to occur in SHRSP, was responsible for the altered decay of MC-PROXYL in the isolated brain as revealed through ESR 2D images. To measure the oxidative stress in the SHR brain under the same experimental conditions as 2D ESR imaging analysis,¹⁹ we performed ESR-CT analysis with MC-PROXYL. Following four minutes of treatment with MC-PROXYL, we measured ESR-CT images (in the x - y plane) of MC-PROXYL in the isolated brains of the SHR and WKY (Figs. 2 and 3). ESR-CT images were reproduced in 16 colors; signals of less than 25% of the maximum signal intensity detected in all slices were regarded as

noise. These ESR-CT images (in the x - y plane) show that the decay of MC-PROXYL in the isolated SHR brain was faster than that in the WKY brain. From ESR-CT analysis (in the x - y plane; Figs. 2 and 3), the rapid decay region may be located in the cerebral cortex of the isolated brain of the SHR. Similar images were obtained in repeated imaging experiments ($n = 4$) on the distribution of MC-PROXYL in the isolated brain of WKYs and SHRs. These results indicate that oxidative stress, which reportedly occurs in SHRs,^{20,21} was cease to alteration of MC-PROXYL ESR-CT images in the isolated brain (Figs. 2 and 3).

Discussion

Free radicals are thought to play a significant role in the pathophysiology of various diseases. Among the many methods available for determining the generation of free radicals in biological systems, ESR has been recognized as one of the most powerful techniques. During the last decade, significant advances in low frequency, *in vivo* L-band and ESR imaging techniques have provided useful information on oxidative stress in biological systems.^{4-19,25-28} ESR-CT imaging is particularly useful for determining the *in vivo* spatial distribution of free radicals in animals. In the present study, we non-invasively demonstrated images (in the x - y plane) of the nitroxyl spin probe distribution of MC-PROXYL in the head region of a mouse measured with ESR-CT imaging (Fig. 1).

Fig. 2. A typical slice pattern of ESR-CT images (in the x - y plane) showing MC-PROXYL distribution in the isolated brain of a WKY. ESR was measured four minutes after intravenous treatment with MC-PROXYL (isolated 30 s after treatment). To assign the anatomic position, representative ESR-CT images in slices in the x - y plane (2.4 mm left or 2.2 mm right of the midsagittal section of the isolated brain; 0) were derived from the corresponding images. As indicated by the attached color scale (16 colors; white and 100 representing the maximum ESR signal), ESR images were reproduced in 16 colors, and signals of less than 25% of the maximal signal intensity detected in all slices were regarded as noise. Experimental conditions were as described under “Materials and Methods.”

Fig. 3. A typical slice pattern of ESR-CT images (in the x - y plane) showing MC-PROXYL distribution in the isolated brain of an SHR. ESR was measured four minutes after intravenous treatment with MC-PROXYL (isolated 30 s after treatment). Assignment of the anatomic position and the attached color scale of the ESR-CT images are the same as those described in Fig. 2. Experimental conditions were as described under “Materials and Methods.”

3D ESR-CT images constructed from the 2D images also indicated that MC-PROXYL was distributed in the live mouse brain (Fig. 1). Although the resolution of the ESR-CT images was not sufficient to resolve the exact anatomical location of MC-PROXYL, the images suggest that the distribution of MC-PROXYL was generally high in the thalamic and hippocampal regions. Further pharmacokinetic studies will be required to evaluate the exact anatomical distribution of MC-PROXYL in the mouse brain.

We have also demonstrated that the decay of the MC-PROXYL ESR signal in the SHR isolated brain as revealed by ESR-CT (in the *x-y* plane) was faster than that in the WKY brain (Figs. 2 and 3). Furthermore, these results suggest that the distribution of MC-PROXYL decayed more rapidly in the region of the cerebral cortex of the isolated brain of the SHR than in that of the WKY (Figs. 2 and 3). As mentioned previously, it is currently very difficult to perform ESR imaging of the head of a living rat with our ESR-CT imaging system and to obtain exact anatomical information. The wide application of the ESR imaging technique for obtaining high-quality images of lossy biological samples has been limited by several factors, including sensitivity, speed of acquisition, and gradient design and accuracy. The current limitations of biological ESR imaging experiments include the relatively low concentration of endogenous free radicals, the lack of availability of ideal stable spin probes, short relaxation times, slow data acquisition, and drifts in microwave frequency, in addition to the magnitude of the static and gradient fields. We are now developing a novel spin probe, software, and resonator for the ESR-CT imaging system that are intended to overcome the current limitations.

Conclusion

The results of the present study indicate that the ESR-CT imaging employed herein can be a powerful tool for selectively detecting free radicals and monitoring free radical reactions *in vivo*. Quantitative ESR-CT imaging analysis with MC-PROXYL will be useful for clarifying the locations of redox status alter under conditions of oxidative stress in the rodent brain. With the ESR-CT imaging system, we also confirmed a higher degree of oxidative stress in the region of the cerebral cortex of SHR brain than in the WKY brain. Further advances in the instrumentation of ESR-CT imaging and the development of optimized nontoxic spin probes would make this technology even more

promising for the non-invasive assessment of oxidative stress in the brain of rodent disease models *in vivo*.

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