Free Radical Research, May 2006; 40(5): 455–460

Enhanced intraarticular free radical reactions in adjuvant arthritis rats

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Accepted by Professor E. Niki

(Received 5 September 2005; in revised form 21 October 2005)

Abstract

One of the reasons of rheumatoid arthritis (RA) development is widely recognized the relation of free radical reactions in tissue injuries. The aim of this study was to evaluate the location where *in vivo* free radical reactions was enhanced in adjuvant arthritis (AA) model rats using *in vivo* electron spin resonance (ESR)/nitroxyl spin probe technique. The signal decay after intravenous injection of spin probe was enhanced in AA than that in control and suppressed by the pre-treatment of dexamethasone (DXT). Interestingly, the decay in joint cavity occurred prior to paw swelling of AA and suppressed by a simultaneous injection of free radical scavengers, indicating that the enhancement of free radical reactions in joint cavity of AA rats. This technique would be useful tool to determine the location of the enhanced free radical reactions and evaluate the activity of antioxidant medicine with non-invasive real-time measurement.

Keywords: *Arthritis, electron spin resonance, nitroxyl radical, spin probe, rheumatoid, join cavity*

Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are becoming more widely recognized as harmful compounds in tissue injury during rheumatoid arthritis (RA). The contribution of ROS in RA has been confirmed by the *in vivo* effect of antioxidants [1–6]. The administration of superoxide dismutase (SOD) and SOD mimic suppressed the development and severity of collagen-induced [1] or adjuvant arthritis (AA) [2] as animal disease model of RA. The antioxidants such as vitamin C [3], vitamin D [4], vitamin E [5] and *N*-acetyl-L-cystein [6] also reduced biochemical change in arthritic model animals.

The production of free radicals was reported by observing spin adduct signals in a specimen of inflamed rheumatoid synovium using electron spin resonance (ESR)/spin trapping technique [7,8]. By detecting the hydroxylation of aromatic compounds, Kaur et al. [9] demonstrated the generation of hydroxyl radical (·OH) in aspirated knee joint fluids and blood from RA patients. Although a source of ROS such as NADPH oxidase [10,11], xanthine oxidoreductase [12], and cyclooxygenase-2 [13,14] have been reported in various tissues, the time-course and the contribution of ROS generation to RA still remains unclear. However, in *in vivo*, many factors seem to influence the degree of arthritis diseases. In addition, it is thought that severed inhibitory enzymes are produced according to the progress of the disease and the imbalances in these enzymes determine the activity of RA disease. Hence, in order to clarify the role of ROS and RNS in RA disease, it is important to examine the spatial and temporal profiles of ROS and RNS *in vivo*.

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ISSN 1071-5762 print/ISSN 1029-2470 online © 2006 Taylor & Francis
DOI: 10.1080/10715760500440536

Unfortunately, there are no direct measurements for *in vivo* free radical reactions in RA. If *in vivo* free radical generation can be estimated non-invasively, such information can be used in therapeutic and preventive strategies.

ESR spectroscopy is a very selective and sensitive technique for detecting free radicals such as ROS and RNS. *In vivo* ESR spectroscopy has been developed to measure the change in free radicals generation [15,16] and redox status [17,18] in living animals. Since its sensitivity to evaluate directly small amount of endogenous free radical is insufficient in *in vivo*, nitroxyl stable radicals have been used as exogenous spin probes for detecting ROS generation non-invasively. After *in vivo* administration of nitroxyl radicals, the ESR signal associated with these probes decreases as a function of time. The loss of signal is attributed to the conversion of nitroxyl radical to the diamagnetic hydroxylamines via free radical reactions. This non-invasive technique has been used to assess the role of free radical reactions play in various animal models [16–22]. These results clearly demonstrate that *in vivo* ESR technique can be very useful to estimate *in vivo* free radical reactions during oxidative stress.

In this study, we applied, for the first time, *in vivo* ESR experiments for the evaluation *in vivo* free radical reactions in joint cavity or peripheral blood vessel of living AA rats. The *in vivo* free radical reactions were non-invasively estimated in real time during AA.

Materials and methods

Chemicals

3-Carbamoyl-2,2,6,6-tetramethyl-pyrrolidine-1-oxyl (carbamoyl-PROXYL) was purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Pentobarbital sodium (50 mg/ml), heat-killed *Mycobacterium butyricum*, and heavy mineral oil were from Dinabot (Osaka, Japan), Difco Laboratories (Detroit, MI), and Sigma (St Louis, MO), respectively. Cu/Zn superoxide dismutase (Cu/Zn-SOD, 4000 U/mg), catalase (6700 U/mg), and dexamethasone (DXT) were from Wako Pure chemicals Industries Ltd. (Osaka, Japan). Carbamoyl-PROXYL was dissolved in distilled water at a concentration of 300 mM and stocked at -20°C until use.

Induction of adjuvant arthritis

Female Sprague-Dawley (SD) rats (4-weeks old) were purchased from Seac Breeding Company (Fukuoka, Japan) and were fed a standard diet (MF, Oriental Yeast Co. Tokyo, Japan) and tap water *ad libitum*. Arthritis was induced by the injection of 0.1 ml of adjuvant into right footpad of rats. To the group of control rats, only vehicle was injected into right footpad. DXT was dissolved in 0.5% of carboxymethylcellulose and given orally at a daily dose of 0.1–0.5 mg/kg body weights to rats. The control group was given vehicle only. Change of body weight and paw swelling, were estimated as clinical evaluation of various days after adjuvant injection. Paw swelling was measured by the thickness or volume of each paw.

All procedures and animal care were approved by the Committee on Ethics of Animal Experiments, Graduate School of Pharmaceutical Sciences, Kyushu University, and were conducted according to the Guidelines for Animal Experiments of Graduate School of Pharmaceutical Sciences, Kyushu University.

ESR measurements

Rats were anesthetized with intrapenitoneal (i.p.) injection of pentobarbital sodium (50 mg/kg body weight) and fixed on a home-made holder. Carbamoyl-PROXYL solution was injected into either tail vein (300 mM, 1.0 ml/kg body weight) or intra-articular region (10 mM, 30 μl), and then ESR spectra were recorded at regular intervals for the left articular region with an ESR spectrometer (JEOL, JES-RE-3X) having an L-band microwave bridge (ES-LBIC) and a loop-gap resonator (diameter 33 mm and length 5 mm, JEOL, Japan). The microwave frequency was 1.1 GHz and its power was 1.0 mW. The amplitude of the 100 kHz field modulations was 0.1 mT. The external magnetic field was swept between 35 and 45 mT at a scan rate of 5 mT/min. Cu/Zn-SOD or catalase was injected into articular together with the probe.

The signal decay rate was determined from the semilogarthmic plot of signal intensity as a function of time after the probe injection as described previously [15].

Statistical analysis

All results are reported as the mean ± standard error (S.E.). Statistical significance was analyzed by either Student's *t*-test or Dunnett's test. The StatView (SAS Institute Inc., version 5.0) was utilized for all the statistical analyses.

Results

Symptoms of adjuvant arthritis rats

Induction of AA was confirmed with the change of body weight and paw swelling of rats (Figure 1). All rats in the adjuvant group showed typical symptom for AA, and significant suppression in body weight gain compared to control group 10 days after adjuvant administration. The paw thickness of immunized right foot increased remarkably on day 3, reaching maximum level, while the unimmunized left foot was not swollen until day 7 and then increased gradually.

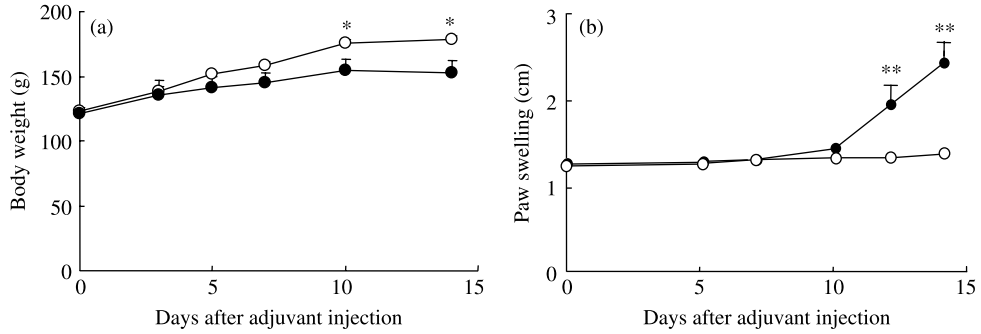

Figure 1. The change of body weight gain (a) and paw swelling (b) of rats. Opened circle: vehicle treated rats. Closed circle: Adjuvant treated rats. Values are expressed as mean ± S.E. of 4–6 rats. Significant differences are $\star P < 0.05$ and $\star\star P < 0.01$.

The slight swelling was observed in the paw only of vehicle-injected foot.

Change of the enhanced signal decay rate in AA development

The spin probe, carbamoyl-PROXYL, was injected into tail vein of rat on day 14 after adjuvant administration and then ESR spectrum of the probe was observed at the articular region of left foot. Figure 2(a) shows the typical ESR spectrum of the probe, which exhibits an isotropic triplet. The ESR parameters, hyperfine splitting constants and *g*-value from the ESR spectra indicated that the probe located mainly in water phase of peripheral blood vessel at articular region but not in adipose tissue. The signal intensity 10 min after the probe injection was smaller than that after 1 min (Figure 2(a)). The logarithms of the signal intensities in control and AA rats were plotted against time after spin probe injection (Figure 2(b)). Straight lines were obtained in both semi-logarithmic plots, indicating that both signal decays obey first-order kinetic equation as described previously [16]. The slope of the signal decay was steeper in AA rat than that in control one. The both decay rate of the probe, which was obtained from the initial slope, significantly increased in AA group than that in control one on day 14 (Figure 3(a)). In previous reports, the oxidative stress caused the enhanced signal decay as a result of free radical reactions and/or ROS generation [15,16]. In order to determine whether or not the enhanced signal decay is related to the development of adjuvant-arthritis rats were treated with anti-inflammatory drug, dexametasone (DXT), and the ESR signal decay was determined on day 14 (Figure 3). The enhanced signal decay in adjuvant rats was significantly suppressed by the treatment of DXT, and the suppressing effect of DXT was in a dose-dependent manner. The treatment completely inhibited the paw swelling on day 14 (Figure 3(b)). To confirm that the enhanced signal decay rate obtained with *in vivo* ESR was due to localized reactions induced by AA, ESR measurements was carried out at different domains, such as breast, abdomen and left articular. Interestingly, any difference was not observed in the signal decay rate between adjuvant and vehicle treated group of AA rats at the breast and abdomen, although significant enhancement was again observed left paw (Figure 4). These results strongly indicate that the enhanced signal decay is associated with the development of AA.

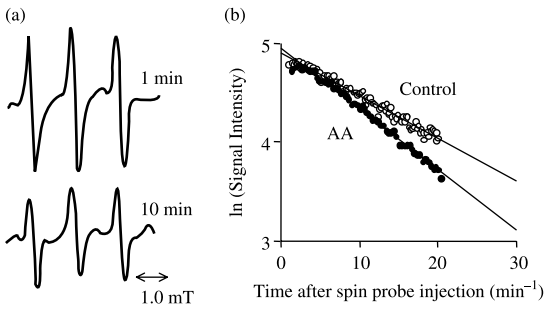

Figure 2. Typical ESR spectra (a) and its signal decay curve (b) of nitroxyl spin probe at left articular region of living rat after intravenous injection. Fourteen days after the injection, rats were anesthetized with i.p. injection of pentobarbital sodium (50 mg/kg body weight) and then carbamoyl-PROXYL solution (300 mM, 1 ml/kg body weight) as a spin probe was injected into tail vein of rats. Immediately after the injection, ESR spectra were obtained at left articular region with an ESR spectrometer (JEOL, JES-RE-3X) under 1.0 mW of 1.1 GHz microwave and 0.1 mT of 100 kHz field modulations.

ROS generation in joint cavity of AA rats

The location and timing of ROS generation must be critically important for the arthritis development. To clarify the spatial and temporal profile of enhanced signal decay rate, nitroxyl probe was injected intravenously or intraarticulaly on various days after adjuvant administration. Figure 5(a) and (b) demonstrated the time course of signal decay rate in peripheral blood vessel and joint cavity of the unimmunized foot after adjuvant injection, respectively. The enhanced signal decay was observed earlier in joint cavity than that in peripheral blood vessel. In joint cavity, the decay rate was enhanced significantly in AA groups on day 7 and was at the same level till day 14, although signal decay on day 10

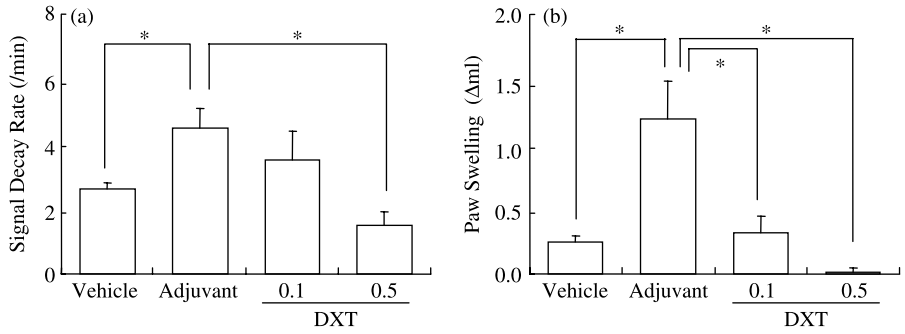

Figure 3. Effect of NSAIDs on signal decay rate ($\times 0.01\ min^{-1}$) of the probe and paw swelling (Δ ml) in adjuvant arthritis rats. (a) Signal decay rate of DXT treated rats. (b) Paw swelling of DXT treated rats. DXT was given orally at a daily dose of 0.1–0.5 mg/kg body weights. The signal decay rate was calculated using the method described in materials and methods. Each value represents the mean ± S.E. $\star P < 0.05$ compared to vehicle treated group. $\star\star P < 0.05$ compared to adjuvant treated group.

in peripheral blood vessel remained as low as that in vehicle treated groups. These results suggested that the enhanced signal decay occurred in joint cavity prior to in peripheral blood vessel. Furthermore, to examine the dependence of ROS on the enhanced signal decay in AA rats, either Cu/Zn-SOD or catalase was simultaneously injected into articular together along with the probe, and then ESR signal was observed in the joint cavity of unimmunized left foot (Table I). The significant enhanced signal decay was also obtained at the joint cavity of AA groups. Simultaneous treatment of Cu/Zn-SOD or catalase in the adjuvant treated groups suppressed significantly the enhanced signal decay to the control level. These results indicate that ROS involving O_2^- and H_2O_2 are generated prior to paw swelling using *in vivo* ESR/nitroxyl spin probe technique and that ROS generation should be contribute to the development of AA.

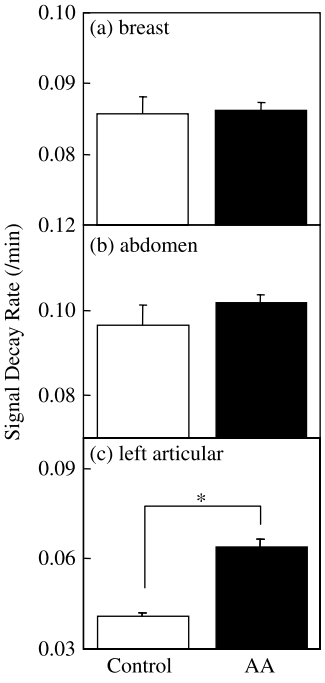

Figure 4. Decay rate of carbamoyl-PROXYL at the breast (a), the abdomen (b) and the left articular region (c) of vehicle (opened bar) and adjuvant (closed bar) treated rats. *In vivo* ESR measurements were done 14 days after adjuvant administration. Values are expressed as mean ± S.E. of 4–6 rats. Significant difference is $\star P < 0.005$.

Discussion

In vivo ESR measurement using nitroxyl radical as a spin probe enables to use *in vivo* free radical production in living animals non-invasively in real time. The development of RA disease is dependent on many factors including ROS at various stages, and these factors involved at each stage would be different. The sources of ROS have been reported as NADPH oxidase [10,11] and xanthine oxidoreductase [12] in various tissues. Generation of ·OH is supported with *in vitro* experiment by detecting stable radical-adducts or hydroxylation products of aromatic compounds in inflamed rheumatoid synovium [9]. The amount of catalytic iron released from synovial fluid ferritin [23] were estimated to be 40% of total iron in the synovial fluids aspirated from inflamed RA knee joints [24]. Therefore, it is thought that these transition metal ion react with H_2O_2 to form ·OH in joint cavity of AA rats. Interestingly, the superficial articular chondrocyte lining the surface of cartilage had metabolic differences from deep cells, including large increment of NO production [25,26]. The articular chondrocyte surface is positioned in the front line attack in inflammatory joint disease, such that it is exposed to various noxious factors contained in the synovial fluids. In addition, we reported that the inducible nitric oxide synthase (iNOS) was expressed in the superficial chondrocytes on days 7–10 [27]. On the contrary, neither significant expression of iNOS in the synoviocyte cell-lining nor accumulation of macrophages in synovium was observed on days 7–10. Hence, the determination of a timing and location of *in vivo* ROS generation is important to consider the therapeutic and preventive strategies.

In vivo ESR measurements using nitroxyl spin probe enable us to estimate non-invasively and real

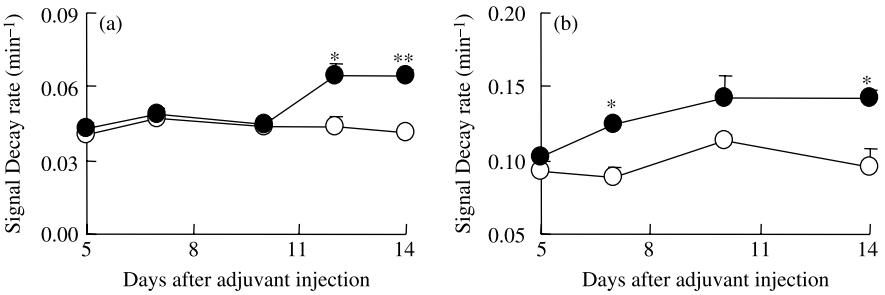

Figure 5. Time course of the enhanced ESR signal decay rate of nitroxyl spin probe at unimmunized foot after adjuvant administration. On the indicated days, the enhanced signal decay rate of unimmunized foot after intravenous (a) or intraarticular (b) injection of spin probe was obtained from the slop of semi-logarithmic plot of signal intensity. Values shown are mean ± S.E. Opened circle: vehicle treated rats. Closed circle: Adjuvant treated rats. The symbol (⋆) and (⋆⋆) indicate significant difference ($P < 0.05$) and ($P < 0.005$), respectively.

timely *in vivo* free radical reaction in living animals. The *in vivo* signal decay of the probe has been reported to relate with physiological reducing capacity [16,20] and also to be enhanced by a various oxidative stress models. In addition, the enhancement was suppressed by *in vivo* treatment of antioxidants and ROS scavengers [16,22,28]. Recently involvement of lipid derived free radical was reported in the initiation of reperfusion after middle cerebral artery occlusion prior to brain edema formation using spin probe having partial membrane permeability and ROS scavengers [15]. *In vitro* experiments have also shown that nitroxyl probe reacts with various free radical species such as superoxide, hydroxyl radical, and peroxyl radicals [29]. These evidences strongly suggest that nitroxyl probe is very susceptible to free radical reactions involved in oxidative damage and that the enhanced signal decay of nitroxyl probe is related with ROS generation.

In the present study, carbamoyl-PROXYL was used as a spin probe to examine free radical reactions in articular region of rat. The signal decay was significantly enhanced in AA group than that in control one. Enhanced signal decay was suppressed by administration of DXT, indicating the association with the development of arthritis. Enhancement of free radical reactions involving ROS in joint cavity occurred prior to that in peripheral blood vessel and the subsequent development of edema. This enhanced signal decay in joint cavity was inhibited by simultaneous treatment with Cu/Zn-SOD or catalase. These results strongly suggested that the enhanced signal decay observed in AA rats was caused by the generation of O_2^- and H_2O_2. Previous our report indicated that concomitant nitrotyrosine residue production, which is the footprint of NO and peroxynitrite ($ONOO^-$) generation, was observed on day 10 [27]. Chemical data suggested that the interaction of O_2^- and NO formed more cytotoxic $ONOO^-$ [30]. The considerations discussed above suggested that high concentrations of ROS and RNS might interact at the articular surface in inflammatory arthritis, resulting in an increase in oxidative damage within the joint cavity prior to the development of edema.

In conclusion, we succeeded in non-invasive and real-time measurements of *in vivo* free radical reactions in AA rats, by observing the enhanced signal decay of the spin probe with *in vivo* ESR spectroscopy. The signal decay was enhanced in AA rats than that in vehicle treated rats, which was inhibited by anti-inflammatory drug. Enhanced signal decay rate in joint cavity caused prior to in peripheral blood vessel and the development of paw swelling, which was suppressed by Cu/Zn-SOD or catalase simultaneous injection with spin probe. These results revealed that the generation of ROS was enhanced in the joint cavity at first prior to paw swelling. This technique should become widely applicable for the determination of free radical reactions involved in oxidative stress.

Table I. Effect of Cu/Zn-SOD or catalase on signal decay rate of the probe in adjuvant arthritis rats ($k \times 0.01\,min^{-1}$).

Treatment dose	Exp. 1		Exp. 2	
	Day 7	Day 14	Day 7	Day 14
Vehicle	13.7 ± 0.7 (4)	13.9 ± 0.6 (4)	12.7 ± 0.7 (4)	10.9 ± 1.2 (4)
Adjuvant	16.7 ± 1.4 (4)⋆	19.7 ± 2.0 (4)⋆⋆	15.9 ± 1.3 (4)⋆	18.7 ± 1.6 (4)⋆⋆
Cu/Zn-SOD	10.1 ± 0.9 (3)⋆⋆	12.0 ± 1.9 (4)⋆⋆	–	–
Catalase	–	–	12.9 ± 1.9 (3)⋆	12.8 ± 1.0 (3)⋆

Either bovine Cu/Zn-SOD (10 U/rat) or catalase (200 U/rat) was simultaneously injected with the probe into the joint cavity of the left foot of rats. Values for the signal decay rate are given as the mean ± S.E. The number in parenthesis and the symbol indicate the experiment number and significant difference (⋆$P < 0.05$, ⋆⋆$P < 0.01$).

Acknowledgements

This work is supported in part by Grants-in-Aid of Scientific Research, for Research in Priority Areas, for Cooperative Research, for General Science Research, and for Developmental Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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