Free Rad. Res., Vol. 35, pp. 245–255
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An Electron Spin Resonance Study on Alkylperoxyl Radical in Thin-Sliced Renal Tissues from Ferric Nitrilotriacetate-Treated Rats: the Effect of α-Tocopherol Feeding

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Accepted by Prof. N. Taniguchi

(Received 15 January 2001; In revised form 6 February 2001)

Formation of excess free radical causes cellular oxidative stress, which has been shown to be associated with a variety of pathologic conditions. While electron spin resonance (ESR) spectroscopy has been the only method to demonstrate the presence of free radicals, its application to tissue samples has been challenging. We report here the successful ESR detection in thin-sliced fresh tissues or frozen sections in a rat model. Ferric nitrilotriacetate (Fe-NTA) induces oxidative renal tubular damage that ultimately leads to high incidence of renal carcinoma in rodents. Twenty minutes after administration of 5 mg iron/kg Fe-NTA to rats, a thin-slice of the kidney was mounted on a tissue-type cell and analyzed by ESR spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). An ESR signal from alkylperoxyl radical adduct was obtained, and the signal was inversely proportional to renal α-tocopherol content which was modulated through diet. Furthermore, we undertook *ex vivo* study using frozen sections. Fe-NTA (1 mM) was added to a rat kidney frozen section for 10 min. After washing the specimen was mounted on a tissue-type cell and analyzed with ESR spin trapping using DMPO. Alkylperoxyl radical signal was dependent on thickness, incubation time and renal tissue levels of α-tocopherol, and was reduced by preincubation with catalase or dimethyl sulfoxide but not with α-tocopherol outside tissue. This versatile method facilitates identification of free radicals in pathologic conditions, and may be useful for selection of antioxidants.

Keywords: Electron spin resonance, tissue ESR, kidney, ferric nitrilotriacetate, peroxyl radical, α-tocopherol

INTRODUCTION

Free radicals are defined as any chemical species that contain one or more unpaired electrons. Production of excess free radical induces oxidative

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stress in cells, which has been believed to play a central role in a number of pathologic conditions such as atherosclerosis, carcinogenesis,[1] diabetes mellitus,[2] drug toxicity (e.g. doxorubicin, bleomycin), radiation damage, neurodegenerative diseases (e.g. Alzheimer's disease, Parkinson's disease), and reperfusion injury.[3] During the last decade, it became clear that oxidative stress works for two completely different directions, namely either for proliferation or death, which depends on its level and probably cell type as well as the responsible free radicals.[4–6]

Recently, a large number of efforts are in progress to select novel antioxidants from natural as well as synthesized chemicals to be used for prevention and treatment of free radical-associated diseases. The evaluation is dependent on the three methods; biochemical, morphological, or electron spin resonance (ESR) method. Measurement of thiobarbituric acid (TBA)-reactive substances has been most commonly used.[7] Recently there are also emerging new markers such as isoprostanes.[8] We have worked on the development of immunohistochemical methods using antibodies against products of free radical reactions (e.g. 8-hydroxy-2′-deoxyguanosine, 4-hydroxy-2-nonenal-modified proteins).[9–11,5]

While ESR spectroscopy has been the only method to demonstrate unpaired electrons and frequently used in *in vitro* evaluation of antioxidants and also for oxidative burst of neutrophils, tissue has been a challenging sample. Although tail of living mouse was used for the detection of nitric oxide by means of iron-dithiocarbamate trapping method,[12] conventional spin trapping method has not been used. We report here ESR spin trapping with 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) in thin-sliced fresh tissues or frozen sections, and its application to a drug-induced renal injury model. The animal model we used in the present study is ferric nitrilotriacetate (Fe-NTA)-induced renal carcinogenesis in rats. This is one of the most intensively investigated models among oxidative stress-induced carcinogenesis. Its detailed data is reviewed elsewhere.[1,13] Our recent observations on this model include the identification of $p15^{INK4B}$ and $p16^{INK4A}$ tumor suppressor genes as one of the major target genes[14] and of 20 independent transcripts that revealed significant change in expression during carcinogenesis.[15]

α-tocopherol is one of the principal endogenous antioxidants, which is believed to defend cells and tissues from free radical damage. The objective of the present study was to establish an application of ESR method to dissected fresh tissues, and to determine whether the modulation of endogenous α-tocopherol levels in tissues through the diet could alter the generation of free radicals catalyzed by iron.

MATERIALS AND METHODS

Animals

Five-week-old male specific-pathogen-free Wistar rats (Shizuoka Laboratory Animal Center, Shizuoka) were purchased. They were kept in stainless steel cages in an air-conditioned room (22–24 °C) with a light/dark cycle of 12 h each and given semisynthetic basic diet AIN-76 (Funabashi, Chiba)[16,17] with or without α-tocopherol supplementation as follows; (1) α-tocopherol deficient diet (vitamin E (−) group); (2) α-tocopherol sufficient diet containing 20 mg/kg diet of DL-α-tocopheryl acetate as a control (vitamin E (+) group); and (3) α-tocopherol-supplemented diet containing 500 mg/kg diet of DL-α-tocopheryl acetate (vitamin E (++) group). Dietary α-tocopherol modulation was employed because its antioxidant activity has been well established.[3,18] Animals were subjected to ferric nitrilotriacetate (Fe-NTA) administration after a four-week controlled diet. Each experimental group consisted of eight animals. The animals were given deionized water (Millipore Japan, Osaka) *ad libitum* during the experiments.

Chemicals

Ferric nitrate enneahydrate, sodium carbonate, superoxide dismutase, ethanol and dimethyl sulfoxide (DMSO) were from Wako (Osaka, Japan); nitrilotriacetic acid disodium salt and 2,6-*di-tert*-butyl-*p*-cresol (BHT) were from Nacalai Tesque Inc. (Kyoto, Japan). Catalase, diazabicyclo[2,2,2]octane (DABCO) and α-tocopherol were purchased from Sigma (St. Louis, MO). All the chemicals used were of analytical quality; deionized water was used throughout. Fe-NTA solution was prepared immediately before use as previously described.[9] Fe-NTA of 5 mg iron/kg body weight was injected intraperitoneally into the animals for fresh tissue-ESR experiments. For *ex vivo* frozen section-ESR experiments, Fe-NTA solution was diluted with 10 mM *N*-[2-hydroxyethyl]piperazine *N'*-[2-ethanesulfonic acid] (HEPES) saline solution, pH 7.4.

Fresh Tissue-ESR Experiments

Animals were killed 20 min after intraperitoneal injection of 5 mg iron/kg Fe-NTA. Our previous experiments showed that this is the earliest time after Fe-NTA administration when we can find the significant difference in TBA-reactive substances.[19] After immediate removal, a half of one kidney was used for determination of TBA-reactive substances and the other half for histological evaluation by hematoxylin & eosin staining after fixation with 10% neutral formalin. The other kidney was used for fresh tissue-ESR experiments. Transverse sectioning at 245-μm thickness near the center of the kidney was done by the simultaneous manual use of two razor blades (#FAS-10, Feather, Osaka). Kidney was placed on ice for repeated use within 1 h. The thin section was placed on a tissue-type quartz cell (LTC-10, Labotec, Tokyo) to which 10 μl of 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO, Labotec) was applied without dilution. ESR signals were obtained with an FR-30 ESR spectrometer (JEOL Ltd, Tokyo). Instrument settings were as follows: standard first-derivative mode with 100 kHz modulation, a modulation amplitude of 0.079 mT; scanning width 10 mT, center magnetic field 339.5 mT; microwave power and frequency of 4 mW/9.425 GHz; scanning time of 2 min with a time constant of 0.30 sec at temperature 290.0 K. Relative height of the lowest field line against an internal standard, $Mn_{(3)}$, was recorded. The obtained relative amplitude was divided by protein amount of the thin-sliced kidney which was determined by BCA method (Pierce, Rockford, IL) for calibration. Duplicate measurement was performed for each kidney, and three animals were used for each group. The sample cell was sequentially cleaned after measurement with 50% methanol/50% chloroform, 100% ethanol and 70% ethanol, and dried each time.

Ex Vivo Frozen Section-ESR Experiments

Kidney of animals fed with α-tocopherol-regulated diet as described above was snap-frozen in Tissue-Tek optimum cutting temperature compound (Miles Inc., Elkhart, IN) with dry ice-cooled acetone and cut with a cryostat (HM500-OM, Carl–Zeiss, Tokyo) at 30 μm thickness. Three animals were used for each experimental group. A half of one kidney was used for determination of α-tocopherol concentration and the other half for histological evaluation. Serial frozen sections were obtained, and protein concentration was determined by BCA method. They were stored at −80 °C until use. The sections were completely dried with a drier at room temperature immediately before use. Fe-NTA solution (200 μl) of determined concentration prepared immediately before use was applied to the section on a glass slide in a moisture chamber, and incubated for different period of time up to 40 min. In standard experiments, the conditions of 1 mM Fe-NTA and 10 min-incubation at room temperature were employed. After the incubation, the section was washed twice in 10 mM HEPES saline solution, pH 7.4, and excess buffer solution around the frozen section was removed

by Kimwipe wipers S-200 (Crecia, Tokyo). Then, the frozen section was removed from the glass slide with Gilson yellow tip, and placed on the tissue-type cell to which 10 μl of DMPO was applied. In the inhibition experiments, antioxidants were added to this system, and compared with the system without the antioxidants. Duplicate measurement was done for each kidney. The instrumental condition and analysis for ESR signal were the same as fresh tissue-ESR experiments.

TBA-Reactive Substances Determination

TBA-reactive substances were determined according to the method of Buege and Aust[7] with slight modification. To prevent additional chromophore formation during the assay, 0.1% BHT was added to the reaction mixture.

Determination of α-Tocopherol

The content of α-tocopherol in the kidney was determined with the high performance lipid chromatography method.[20]

Determination of γ-Glutamyl Transpeptidase (γ-GTP) Activity

γ-GTP activity was determined as described.[21]

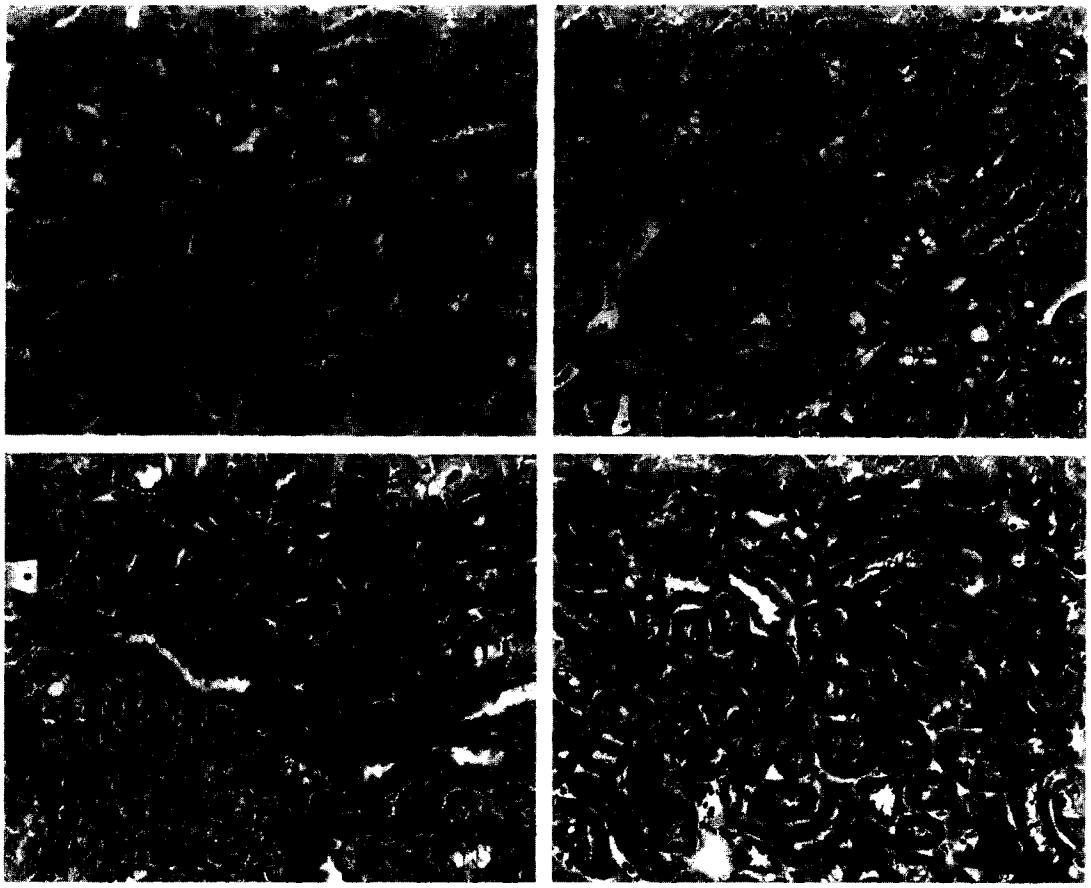

FIGURE 1 Histology of rat kidney after intraperitoneal Fe-NTA administration of 5 mg iron/kg. (A) Untreated control rat; (B) Vitamin E-deficient rat. Nuclear pyknosis is observed in most of the renal proximal tubular cells; (C) Vitamin E-sufficient rat. Nuclear pyknosis is observed in approximately half of the renal proximal tubular cells (arrows); (D) Vitamin E-supplemented rat. No remarkable change is observed (Hematoxylin & eosin staining; bar, 100 μm).

Statistical Analyses

Statistical analyses were performed using an unpaired *t*-test, which was modified for unequal variances when necessary.

RESULTS

Modification of Renal α-Tocopherol Concentration by Diet

Renal content of α-tocopherol was $0.423^{***} \pm 0.116\,\mu g/g$ for vitamin E (−) group, $8.923 \pm 0.876\,\mu g/g$ for vitamin E (+) group and $24.13^{**} \pm 2.623\,\mu g/g$ for vitamin E (++) group (means ± SEM, $N = 3$; $^{**}p < .01$ and $^{***}p < .001$ vs vitamin E (+)). Microscopic analysis showed no difference in the histology of kidney among the three groups (data not shown). Renal γ-GTP activity was 1.88 ± 0.23 U/mg protein for vitamin E (−) group, 2.24 ± 0.09 U/mg protein for vitamin E (+) group and 1.99 ± 0.29 U/mg protein for vitamin E (++) group (means ± SEM, $N = 3$; no significant difference among the three groups).

Renal Lipid Peroxidation After Fe-NTA Administration

Renal TBARS after intraperitoneal Fe-NTA administration was $13.50^{*} \pm 0.85$ nmol malondialdehyde (MDA)/100 mg protein for vitamin E (−) group, 9.25 ± 0.44 nmol MDA/100 mg protein for vitamin E (+) group and $6.79^{*} \pm 0.47$ nmol MDA/100 mg protein for vitamin E (++) group (means ± SEM, $N = 3$; $^{*}p < .05$ vs vitamin E (+)). Twenty minutes after administration of Fe-NTA, nuclear pyknosis was noted only in the kidney of vitamin E (−) and (+) groups. Kidney of vitamin E (−) showed nuclear pyknosis in the majority of proximal tubules whereas that of vitamin E (+) revealed only focal changes (Figure 1(A, B, C).

Fresh Tissue-ESR Experiments

Fresh renal tissue of vitamin E (+) group from two rats was first used to evaluate the background signal level. The signal was practically not detectable up to 30 min after application of DMPO (Figure 2). Then, fresh renal tissue obtained from Fe-NTA administered rats was evaluated. Although Fe-NTA itself is ESR-active,[22] fresh-tissue showed ESR signals different from that of Fe-NTA alone (Figure 3(A,B)). Hyperfine coupling constants of this signal were analyzed as one nitrogen, $a_N = 1.361$ mT and one hydrogen; $a_H = 1.118$ mT. This signal was identified as alkylperoxyl radical (oleic, linoleic, linolenic or arachidonic)[23] adduct of DMPO based on spectrum simulation (Figure 3C) and the following reasons; (1) this signal was obtained in organic solvent, but the solvent was not toluene;[23]

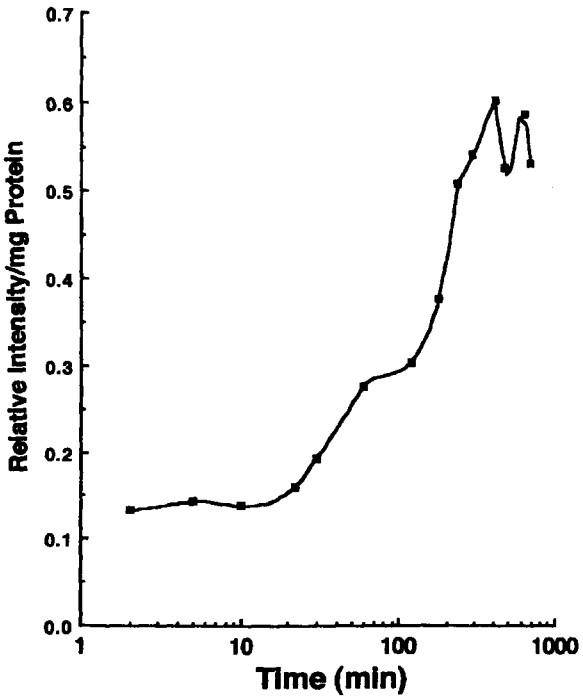

FIGURE 2 Relative intensity of ESR signal on untreated renal thin-slice of 245 μm from rats fed with vitamin E-sufficient diet. Refer to materials and methods for details. Each point is a means of duplicate experiments. Compare this with Figure 4.

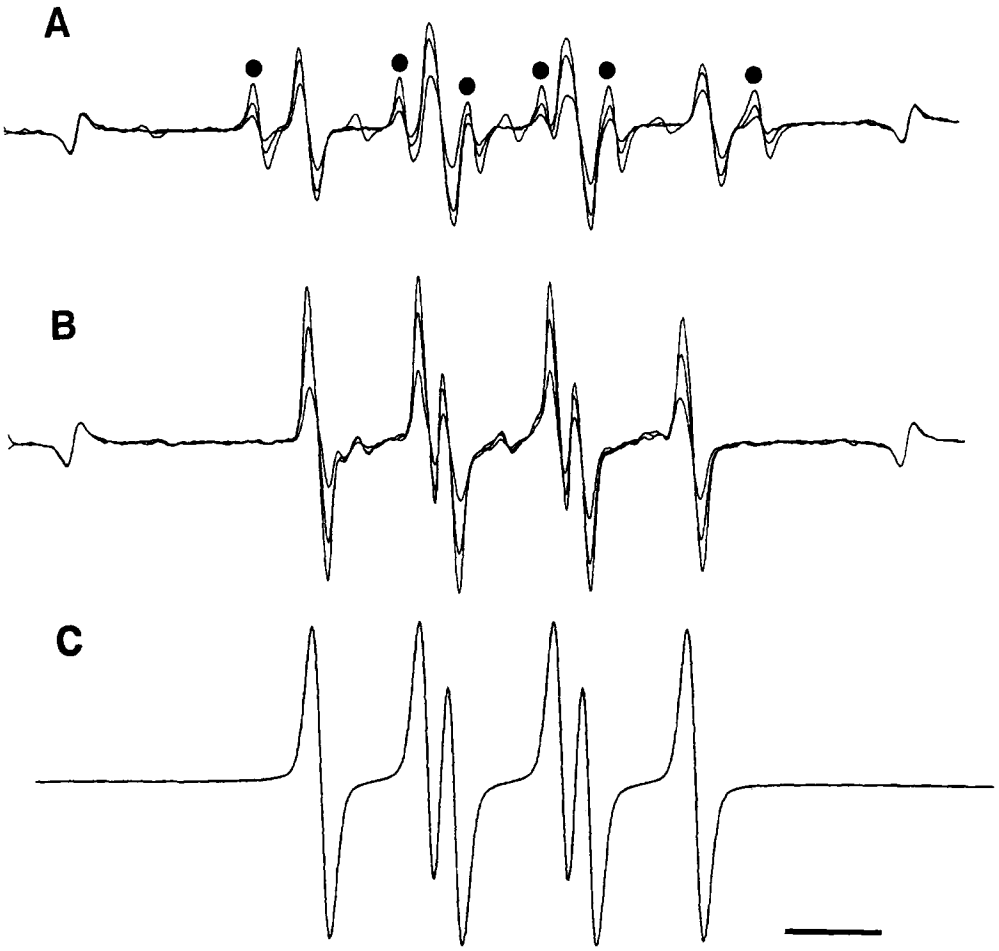

FIGURE 3 ESR spectra observed on (A) 1 mM Fe-NTA solution, (B) renal thin-slice of 245 μm from a rat that were fed with vitamin E-sufficient diet and received intraperitoneal injection of 5 mg iron/kg Fe-NTA 20 min before; and (C) simulation of the ESR spectra shown in B. (A)(B), signals at 1, 4, 8 min after application of DMPO are simultaneously shown. Filled circle, decreasing signal; (C) Hyperfine condition of $a_N = 1.361$ mT and $a_H = 1.118$ mT was used for the simulation (bar, 1 mT).

(2) non-aqueous, solid portion was responsible for the signal; and (3) high concentration of DMPO was necessary for spin trapping. Furthermore, the signal intensity was inversely correlated with the levels of α-tocopherol (Figure 4(A, B)).

Ex Vivo Frozen Section-ESR Experiments

These experiments were undertaken to evaluate whether ESR can be applied to frozen sections in *ex vivo* condition, and to chracterize this reaction in reference to renal lipid peroxidation induced by Fe-NTA *in vivo*. Firstly, we confirmed that compounds used for frozen sections, or vacuum grease (Labotec) used for closing the tissue-type cell do not give any significant signal. Then, we examined the frozen sections (Vitamin E (+) group) treated with 10 mM HEPES saline solution instead of Fe-NTA. These sections did not show any significant signal (data not shown). However, when frozen section was treated with Fe-NTA, followed by extensive washing with HEPES saline buffer, an ESR signal of alkylperoxyl radical adduct of DMPO was obtained. This was basically the same signal obtained in fresh-tissue ESR (data not shown).

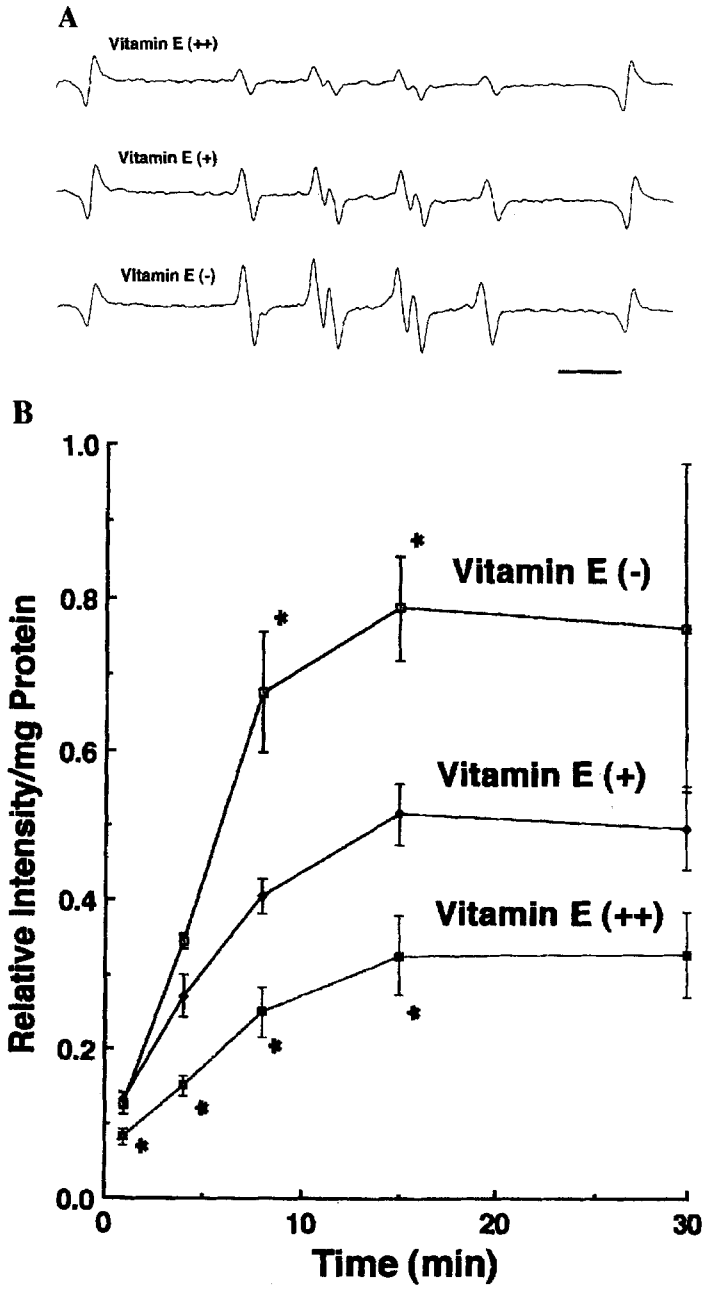

FIGURE 4 Relative intensity of ESR signal on renal thin-slice of 245 μm from a rat that were fed with vitamin E-modified diet and received intraperitoneal injection of 5 mg iron/kg Fe-NTA 20 min before. (A) ESR spectra; (B) Relative intensity of ESR signal. Refer to materials and methods for detail. Compare this with Figure 2 as a control for untreated thin-sliced kidney (bar, 1 mT).

Furthermore, this reaction was dependent on the concentration of Fe-NTA applied, the incubation time with Fe-NTA, and the thickness of frozen sections. Alkylperoxyl radical signal increased according with the concentration of Fe-NTA up to 10 mM (Figure 5A). We evaluated the incubation time up to 40 min. The signal intensity reached at the maximal level after 10 min incubation (Figure 5B), and was proportional to the thickness of frozen section (Figure 5C).

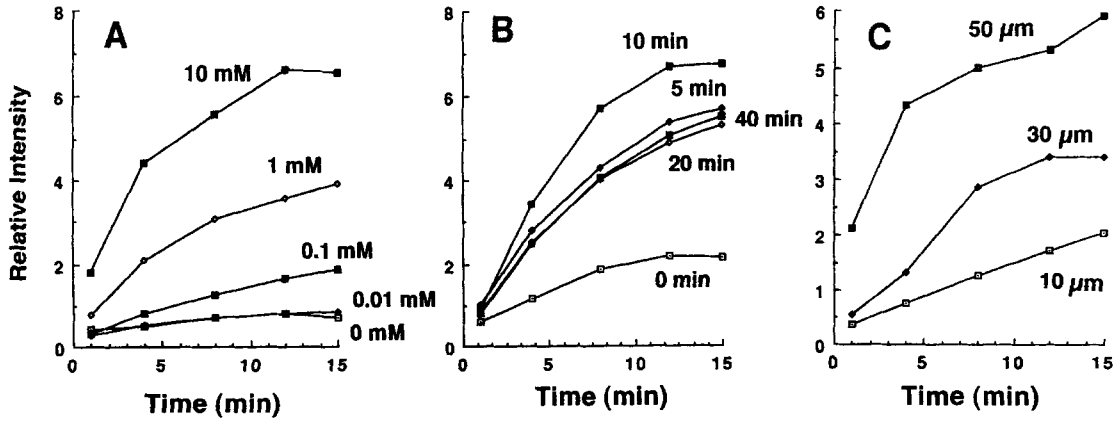

FIGURE 5 Characterization of *ex vivo* frozen section-ESR experiments. Dependency of the ESR signal on Fe-NTA concentration, incubation period with Fe-NTA and thickness of the frozen section is shown. Refer to materials and methods for detail.

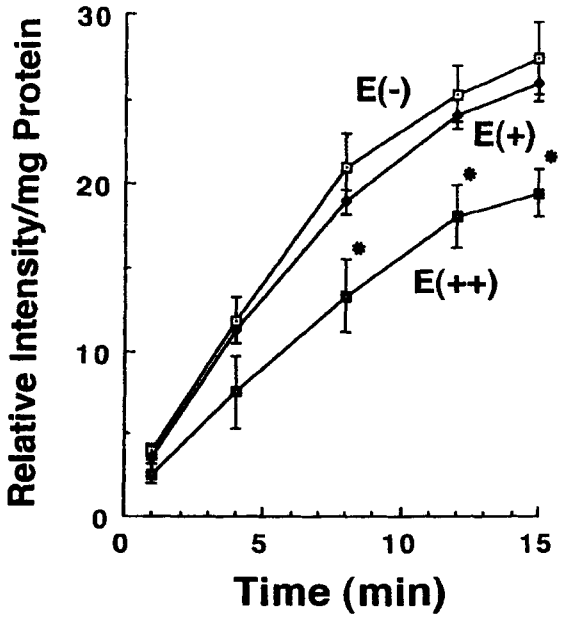

FIGURE 6 Relative intensity of ESR signal on Fe-NTA-applied frozen sections from rats fed with vitamin E-modified diet. Refer to materials and methods for detail.

Based on these results, we have determined the standard condition as 1 mM Fe-NTA, 10 min incubation and 30 μm thickness. Relative free radical amount in the frozen section was calculated with dividing ESR intensity by protein content of the tissue. We recorded the signals of renal frozen sections obtained from animals fed with different vitamin E-concentration modified diet. There was an inverse association between the vitamin E level and the ESR signal (Figure 6). No significant signals were observed in the liquid fraction of the content of the tissue-type cell, suggesting that we looked at tissue-bound radicals.

We then studied the effect of several antioxidants on the spin trapping reaction in isolated tissues. Data for the highest concentration of antioxidants applied is summarized in Table I. There was an inhibitory effect by catalase and DMSO. However, ethanol, BHT and α-tocopherol revealed no significant effect. Conversely, SOD and DABCO enhanced the signal intensity.

TABLE I

Antioxidants	Inhibition (%)
SOD (500 unit/ml)	−9.6
Catalase (333 unit/ml)	23.2
DABCO (0.17 M)	−52.8
Ethanol (16.7%)	6.5
DMSO (16.7%)	30.3
BHT (1.67 mM)	1.6
α-tocopherol (1.67 mM)	0

Refer to materials and methods section for detail.

DISCUSSION

Oxidative stress by free radical is involved in a variety of pathologic conditions including carcino-

genesis. Our previous studies using a Fe-NTA-induced rat renal carcinogenesis model[1] include increased levels of aldehydes including 4-hydroxy-2-nonenal and malondialdehyde,[9,24] DNA base modifications such as 8-oxoguanine,[25] and thymine-tyrosine cross-link.[26] These molecules are considered to be the products of free radical reactions, and often regarded as indirect evidence of the free radical involvement. Therefore, we undertook to obtain more direct evidence of the primary reaction in the kidney of this model after Fe-NTA administration.

ESR spin trap technique is a robust and so far the only method to identify free radicals. However, it has been applied to only aqueous solution or suspension of biological samples. In NO trapping method using Fe-dithiocarbamate complex as a spin trap, ESR signals from tissue sections have been recorded.[12] However, in conventional *in vivo* spin trapping, tissues were subjected to extraction with organic solvent and ESR signals of the extract was recorded. Such indirect method may cause a loss of free radicals or spin adducts during the extraction. Direct ESR observation from tissues is preferable, but one of the major obstacles for this has been the absence of an appropriate ESR-cell for tissue application. A newly developed cell for tissue (LTC-10, Labotec, Tokyo Japan) was used in the present study. This sample cell has a groove with the size of 40 mm × 5 mm × 0.67 mm for holding the tissue.

In the fresh-tissue ESR experiments, we have identified the obtained signal as alkylperoxyl radical based on computer spectral simulation. The intensity of the signal was inversely associated with renal vitamin E content, which was consistent with our previous data on lipid peroxidation.[18] Since it is known that Fe-NTA itself is a free radical (Figure 3A),[22] we performed the proper control experiments as described in the results section. This method has an advantage over other methods because it took only 15 min to perform.

We also determined whether this method is applicable to *ex vivo* conditions. Previously, we have used *ex vivo* methods to show the association of free radical damage with γ-GTP activity,[27] or with distribution of an antioxidant molecule, thioredoxin.[28] Unexpectedly, the same ESR signal as that of fresh tissue-ESR experiments was obtained. We selected optimal conditions based on the dependence of this reaction on the concentration of Fe-NTA applied and thickness of frozen section. When a frozen section was incubated for more than 10 min, the signal decreased. We speculate that extensive tissue damage after prolonged exposure to Fe-NTA lowers the generation of alkylperoxyl radical from tissue. These conditions were used to determine the dependence of signal level on the vitamin E content of the kidney section. Similarly to fresh tissue experiment, the signal decreased as the vitamin E content in frozen tissue increased. We showed previously that the reaction in the lumina of renal proximal tubules is γ-GTP-dependent.[27] However, in these tissues, the levels of γ-GTP activity were similar, suggesting that vitamin E level is a determinant of the signal level. In the experiments to test inhibitory activity of various antioxidants against DMPO spin trapping reactions, only catalase and DMSO were effective. This means that (1) H_2O_2, but not superoxide, is important for the reaction; and (2) key reaction is occurring in the tissue-bound lipid soluble fraction since free α-tocopherol was not effective (Table I).

We performed additional *ex vivo* frozen section-ESR experiments using kidneys of rats that received oral administration of a flavonoid, αG-rutin. Previously, we have shown that these agents protected from Fe-NTA-induced renal damage.[29] However, in the present experiment, no inhibitory effect was observed in *ex vivo* frozen section-ESR experiments (data not shown). We speculate that chelating activity rather than antioxidant activity is functional in the protection of oxidative renal damage. Therefore, we believe that the application of ESR spin trapping method to tissue will also be helpful for the selection of useful antioxidants. So far, basically

two strategies have been taken for the selection of antioxidants for the use of prevention and therapy of free radical-associated diseases. The first method is based on *in vitro* reactions: certain free radical-generation system (e.g. hydroxyl radical, superoxide, peroxynitrite, etc.) is constructed, and inhibition of the reaction by possible antioxidants is evaluated with markers for oxidative stress. Either pure chemical reaction, tissue homogenate or cultured cells are used. The merit of this strategy is its simplicity, good cost-performance and a small amount of antioxidants to be used for the study. The question here is whether the same reaction occurs *in vivo*. The second approach is based on animal experiments in models associated with oxidative stress. This is a critical test when one wants to demonstrate usefulness of the antioxidants with respect to organ- and disease-specificity. However, the experiments may take a long time and is expensive, and large amounts of antioxidants are necessary for treatment. In such a situation, *ex vivo* frozen section-ESR method may serve as an alternative approach. In the animal experiments, it is important to determine what levels of oxidative stress are to be given for the evaluation. The reason is that antioxidant effect may be obscured if the stress is either too high or too low. For this reason, a large number of animals are usually required. The present method may be helpful in decreasing the number of animals, thus the amount of antioxidants used, because we can modify the oxidative stress on the section by changing the concentration of Fe-NTA or other chemicals that induce oxidative stress. Other variables may also be modified by pretreatment of the animals with chemicals such as inhibitors of certain enzymes.

In conclusion, we have applied either thin-sliced tissue or frozen-sections to ESR spin trapping technique for tissue-bound radicals, which worked well for Fe-NTA-induced oxidative renal injury model. Our methods have advantages in that (1) identification of the responsible free radicals is possible; (2) smaller number of animals is necessary, thus smaller amounts of antioxidants are required; and (3) flexible enough to give many modifications in search of the mechanisms of oxidative damage. Therefore, the present method may contribute to further understanding of free radical-associated pathogenesis and give an alternative method for the evaluation of antioxidants.

Acknowledgements

We thank Dr. Yashige Kotake (Oklahoma Medical Research Foundation, Oklahoma City, Oklahoma) for critical reading of the manuscript, and Ms. Yoshie Fujiwara and Ms. Yoko Tanaka for technical assistance. This work was supported in part by a Grant-in-Aid from the Japanese Ministry of Education, Science, Sports and Culture, and a grant from the Program for Promotion of Basic Research Activities for Innovative Bioscience (PROBRAIN).

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