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In Vivo Measurement of Redox Status in Streptozotocin-Induced Diabetic Rat Using Targeted Nitroxyl Probes

KEN-ICHI YAMADA, DAISUKE INOUE, SHINGO MATSUMOTO, and HIDEO UTSUMI

ABSTRACT

***In vivo* electron paramagnetic resonance (EPR) with nitroxyl spin probes has been used for the evaluation of *in vivo* free radical reactions and redox status in living animals. The aim of this study was to clarify the location of free radical reactions induced by hyperglycemia in osteogenic disorder shionogi (ODS) rats using *in vivo* EPR spectroscopy. Diabetes was induced by intravenous injection of streptozotocin (STZ). The amount of ascorbic acid (AsA) in ODS rats was controlled by feeding AsA-containing water. Fourteen days after STZ injection, blood glucose and plasma malondialdehyde levels in STZ-treated rats significantly increased compared with untreated rats. Signal decay rates of intravenously injected 3-carbamoyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (carbamoyl-PROXYL) (less membrane permeable) and 3-carboxy-PROXYL (membrane impermeable) were enhanced in STZ-treated rats in agreement with the previous reports. The decay rate of 3-acetoxymethoxy-PROXYL (membrane permeable) was significantly enhanced by STZ treatment in AsA-depleted rats, and this enhancement was partially restored to the control value by xanthine oxidase inhibitor, although the rate in AsA-supplemented rats was not changed by STZ treatment. These results suggested that the enhancement of signal decay occurred mainly in the intravascular region in STZ-induced diabetic rats and that AsA depletion induced the enhancement of intracellular signal decay through xanthine oxidase, although it is not clear whether the enhancement of signal decay is the cause or the effect of STZ-induced diabetes.** *Antioxid. Redox Signal.* 6, 605–611.

INTRODUCTION

REACTIVE OXYGEN FREE RADICALS have been reported to be generated in patients with diabetes mellitus (19, 34). Various mechanisms have been postulated for the radical generation in diabetes: (1) glycation of biological substances, (2) alteration of the polyol pathway, (3) activation of NAD(P)H oxidase, and through (4) activated endothelial nitric oxide synthase or (5) xanthine oxidase (8, 10, 16, 18), but the precise location of the radical generation in diabetes remains to be identified.

Non-invasive measurements of the *in vivo* free radical reactions and redox status in living animals are important in understanding physiological/pathophysiological processes under oxidative stress. *In vivo* electron paramagnetic resonance (EPR) techniques using nitroxyl spin probes have been widely utilized to obtain information on free radical reactions (7, 22), redox status (14, 35), and pH level (6, 29). The evaluation of free radical generation is based on the reduction/oxidation reactions of nitroxyl radicals with superoxide in the presence of reducing agents (13, 23), hydroxyl radical (24), and ascorbic acid (AsA) (21, 32), *etc.* The change of EPR signal of a nitroxyl probe injected to animals has been observed as "enhanced signal decay" in various diseases (5, 7, 14, 16, 22, 26, 35). Recently, we demonstrated that the signal decay rate of a 2,2,5,5-tetramethyl-1-pyrrolidinyloxy (PROXYL) derivative was increased in streptozotocin (STZ)-induced diabetes (16, 26). The enhanced signal decay was correlated with blood glucose level and was suppressed by pretreatment with insulin or α-tocopherol and simultaneous injection of superoxide disumutase (27), indicating that the enhanced signal decay was due to reactive oxygen species (ROS) generation *in*

Laboratory of Bio-function Science, Graduate School of Pharmaceutical Sciences, Kyusyu University, Fukuoka, Japan.

vivo. Further, the increased xanthine oxidase activity in plasma was responsible for the enhanced signal decay in STZ-treated rats (16). These results suggested that the generation of free radicals such as superoxide was related to the progress of STZ-induced diabetes.

However, it still remains to be resolved where the signal decay was enhanced in STZ-induced diabetes. The use of various spin probes having different membrane permeability should be helpful to determine the location where the enhanced signal decay occurs. Recently, we succeeded in synthesizing spin probes having different membrane permeability and retention in brain (25, 27, 31). The *in vivo* EPR imaging data elucidated that 3-acetoxymethyl (AMC)-, 3-carboxy-, and 3-carbamoyl-PROXYL had maximal, zero-level, and intermediate membrane permeabilities, respectively, indicating that this characteristic of the three spin probes gives us the information to understand where the nitroxyl probes are in living animals under oxidative stress.

AsA has been reported to relieve the symptoms of diabetes (2, 3, 9). Administration of AsA in rats with diabetes restored the liver malondialdehyde (MDA) level to the control value (2). Osteogenic disorder shionogi (ODS) rats are utilized to evaluate the influence of endogenous AsA because these rats have an inherited defect of AsA synthesis to lack L-gulono-γ-lactone oxidase (EC 1.1.3.8), which catalyzes the last step of AsA biosynthesis (12). The AsA level in ODS rats can be modified by the duration of dietary AsA restriction (28). Supplementation of adequate AsA maintains the AsA concentration in ODS rat liver to the level of Wistar rat liver (33) and gives normal growth and fertility to ODS rats (17). In addition, uric acid and glutathione levels in liver are not altered by the AsA depletion feeding during experimental periods (28). Therefore the ODS rat is suitable to estimate the effect of endogenous AsA on the development of STZ-induced diabetes.

The aim of this study was to clarify the location of enhanced free radical reactions in rats with STZ-induced diabetes, and to determine the effect of AsA alteration on the diabetes and nitroxyl signal decay rate using *in vivo* EPR spectroscopy.

MATERIALS AND METHODS

Materials

STZ was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). AsA, allopurinol, and other chemicals were from Wako Pure Chemical (Osaka, Japan). 3-Carbamoyl-PROXYL and 3-carboxy-PROXYL were from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). AMC-PROXYL was synthesized as described previously (27). Partition coefficients between *n*-octanol and phosphate-buffered saline of nitroxyl spin probes (shown in Table 1) were determined with an X-band EPR spectrometer (JES-RE-1X, JEOL, Akishima, Japan) as described previously (27).

Animals

Four-week-old ODS rats (*od/od*), purchased from Clea Japan (Tokyo, Japan), were housed in a room with a 12-h light/dark cycle and allowed free access to food and water. After the acclimation, ODS rats were supplied with AsA-free feed (CL-2; Clea Japan) and divided into two groups, and for 2 weeks AsA-supplemented and AsA-depleted groups were given autoclaved tap water with and without 1 g of AsA /L, respectively, which is a sufficient amount to maintain normal growth and fertility (17). Diabetes was induced by the injection of STZ (65 mg/kg) via the tail vein on the first day that AsA-depleted feeding was started. Two weeks after STZ treatment and the AsA depletion feeding, blood glucose level was determined as described previously (16).

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.

Determination of plasma and liver AsA and MDA levels

The determination of AsA concentration was performed as described by Behrens and Madere using high-performance liquid chromatography with electrochemical detection as follows (1). Collected blood samples were centrifuged, and the plasma was mixed with an equal volume of 20% metaphosphoric acid, stored in an ice bath for 5 min, and centrifuged at 9,000 *g* for 5 min. Removed liver was homogenized (10% wt/vol) in ice-cold 20% metaphosphoric acid using a Teflon–glass homogenizer, and then the homogenate was centrifuged at 9,000 *g* for 5 min. The supernatants of both samples were diluted to 100 µl with a mobile phase solution, and a 5-µl aliquot of the solutions was injected directly into the high-performance liquid chromatograph (TSK gel ODS-80TsQA, 4.6 mm i.d. × 250 mm; Tosoh Corp., Tokyo), eluted with 80 m*M* sodium acetate buffer (pH 4.8) containing 1 m*M* *n*-octylamine and 0.015% metaphosphoric acid at a flow rate of 0.8 ml/min and room temperature, and detected with an

TABLE 1. BIOPHYSICAL PROPERTIES OF NITROXYL PROBES

	$P_{o/w}$*		
Nitroxyl probe	*Experimental*	*Reference*	*Membrane permeability*
Carbamoyl-PROXYL	0.68	0.68 (30)	Intermediate
AMC-PROXYL	9.49	4.1 (27)	Maximum and retainable in cell
Carboxy-PROXYL	0.0049	0.0047 (30)	Zero

*Partition coefficient between *n*-octanol and phosphate-buffered saline.

amperometric detector (WE-GC glassy-carbon electrode; Eicom Corp., Kyoto, Japan).

The levels of MDA in liver and blood samples were determined according to the methods of Ohkawa *et al.* (20). The concentration of the thiobarbituric acid-reactive products was measured using the absorbance at 532 nm.

In vivo *EPR signal decay of nitroxyl probes*

In vivo EPR measurements were done as described previously (27). Fourteen days after STZ treatments, rats were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg) and fixed on a home-made Teflon holder. Carbamoyl-, carboxy-, or AMC-PROXYL solution (0.27 mmol/kg of body weight) was injected into the tail vein, and then the EPR spectrum was immediately measured at the upper abdomen of the rat using an EPR spectrometer equipped with a 300 MHz microwave power unit and a large-size (70 mm diameter) loop-gap resonator. The microwave power was set at 2.0 mW. The amplitude of the 100 kHz field modulation was 0.1 mT. The external magnetic field was swept from 9.8 to 11.8 mT at a scan rate of 6 mT/min.

Allopurinol, an inhibitor of xanthine oxidase, was administered by intraperitoneal injection (50 mg/kg) to AsA-deficient diabetic ODS rats 2 h before *in vivo* EPR measurements.

Statistical analysis

All results are reported as the mean ± SD. Statistical significance was analyzed by either Student's *t* test or the Bonferroni/Dunn test. StatView version 5.0 (SAS Institute Inc., Cary, NC, U.S.A.) was utilized for all the statistical analyses.

RESULTS

Location of enhanced free radical reactions in rats with STZ-induced diabetes

The induction of diabetes in ODS rats was evaluated 14 days after STZ administration by measuring blood glucose levels. The blood glucose level in AsA-supplemented rats was approximately three times higher in the STZ-treated group than that in the vehicle-treated group (Table 2). Plasma and liver AsA concentrations were not significantly different between rats with and without STZ treatment in this study. The plasma MDA level, an index of lipid peroxidation, was 1.9 times higher in STZ-treated rats than that in vehicle-treated rats, while the liver MDA level was not different between the two groups. These results suggested that STZ-treated rats showed the increase of lipid peroxidation in plasma but not in the intracellular site of liver.

To clarify the location where the free radical generation was enhanced, *in vivo* EPR measurement was performed with nitroxyl probes having different membrane permeability as shown in Table 1. Figure 1 demonstrates typical EPR spectra of the nitroxyl probes at the upper abdomen of rats after intravenous injection. The *in vivo* spectrum of carbamoyl-PROXYL (Fig. 1A) was the same as the previous results (16, 26). Four minutes after intravenous injection, the EPR signal intensities of the nitroxyl probes decreased to 20–30% of initial intensities. The signal decay rates of nitroxyl probes were calculated from the slope of the decay curve of EPR signal intensities (Fig. 1G, H, and I) as described previously (16, 26). The decay rate of carbamoyl-PROXYL was significantly enhanced in STZ-treated rats in agreement with previous results (16, 26) (Fig. 2). In previous reports, the enhanced signal decay observed was almost restored by pretreatment with insulin, α-tocopherol, and the superoxide scavengers, superoxide dismutase and tiron (16, 26). Further, xanthine oxidase activity was related to the enhanced signal decay rate (16). These results suggest that the enhanced signal decay in the hyperglycemic condition is due to *in vivo* generation of ROS, especially superoxide. The signal decay rate of carboxy-PROXYL in STZ-treated rats was increased to 1.2 times of that of untreated control rats. However, AMC-PROXYL did not show any enhancement of signal decay. AMC-PROXYL has the specific characteristic of remaining in the cell after penetration through the cell membrane and hydrolysis. These facts indicate that the *in vivo* signal decay is enhanced in STZ-induced diabetic rats and that the enhanced signal decay

TABLE 2. EFFECT OF STZ INJECTION ON ASA AND MDA LEVELS IN ODS RATS

	Group			
	AsA-supplemented		*AsA-deficient*	
	Vehicle	*STZ*	*Vehicle*	*STZ*
Blood glucose (m*M*)	8.2 ± 1.2	17.7 ± 2.9*	8.3 ± 1.1	18.3 ± 3.7*
AsA				
Plasma (nmol/ml)	14.08 ± 2.85	17.39 ± 5.76	0.47 ± 0.10†	0.42 ± 0.10†
Liver (μmol/g)	0.730 ± 0.231	0.729 ± 0.284	0.045 ± 0.014†	0.026 ± 0.008†
MDA				
Plasma (nmol/ml)	5.70 ± 1.16	10.98 ± 3.82*	6.71 ± 1.16	12.98 ± 5.28*
Liver (μmol/g)	7.01 ± 1.18	7.60 ± 0.71	7.31 ± 1.09	8.26 ± 1.20

ODS rats were supplied with AsA-free food, and AsA-supplemented and AsA-depleted groups were given autoclaved tap water with and without 1 g of AsA/L, respectively, after STZ treatment. All values represent the mean ± SD.

*$p < 0.05$, compared with vehicle-treated rats.

†$p < 0.001$, compared with AsA-supplied rats.

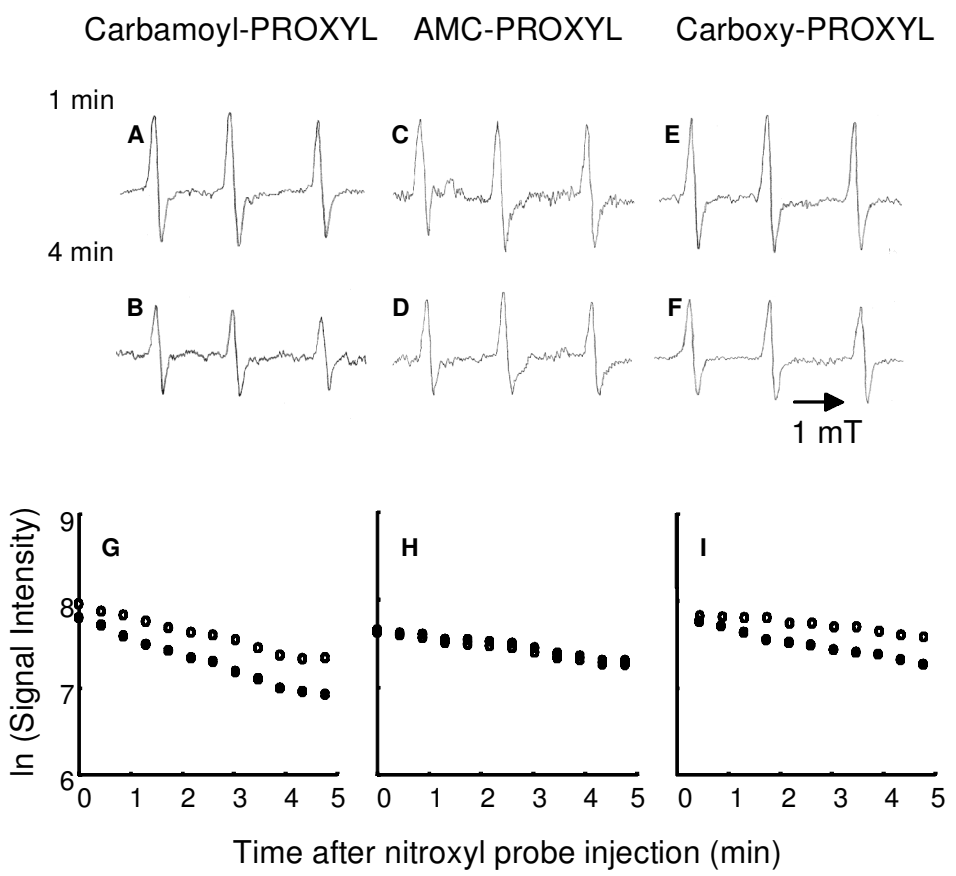

FIG. 1. ***In vivo*** **EPR spectra at the upper abdomen of AsA-supplemented ODS rats after intravenous injection of nitroxyl probe.** The spectra were obtained by using three different nitroxyl probes: carbamoyl-PROXYL (**A**, 1 min; **B**, 4 min), AMC-PROXYL (**C**, 1 min; **D**, 4 min), and carboxy-PROXYL (**E**, 1 min; **F**, 4 min). Also shown are *in vivo* ESR signal decay curves of nitroxyl radicals at the upper abdomen of ODS rats: (**G**) carbamoyl-, (**H**) AMC-, and (**I**) carboxy-PROXYL. Closed circle is STZ-treated group. Open circle is vehicle-treated group. EPR conditions were as follows: microwave frequency, 300 MHz; power, 2 mW; field modulation width, 1 mT; field modulation frequency, 100 kHz.

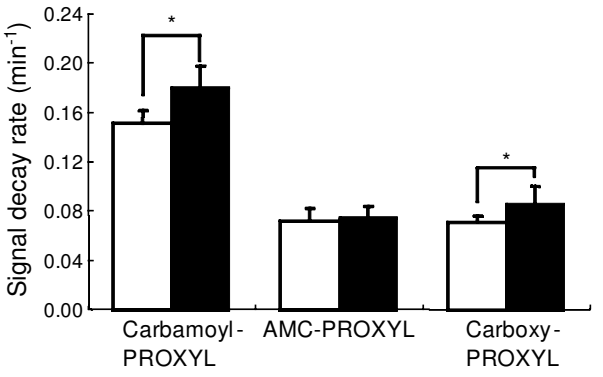

FIG. 2. ***In vivo*** **EPR signal decay rate of AsA-supplemented diabetic ODS rats.** On day 14 after STZ or vehicle administration, the signal decay rate was observed at the upper abdomen of ODS rats using three different nitroxyl probes: carbamoyl-PROXYL, AMC-PROXYL, and carboxy-PROXYL. Open bar is vehicle-treated group. Closed bar is STZ-treated group. Values are mean ± SD (n = 7–9). *$p < 0.05$, statistically significant difference compared with vehicle-injected group.

rate occurred in mainly the intravascular region but not intracellularly. ROS such as superoxide generated in the intravascular region may cause the enhanced signal decay.

STZ-induced diabetes in AsA-depleted rats

Supplementation of AsA exogenously has been reported to relieve the symptoms of diabetes (2, 3, 9) and restore the liver MDA level to the control value (2), indicating the involvement of AsA in diabetes. In this study, we examined the effect of endogenous AsA depletion on the development of STZ-induced diabetes. AsA-deficient rats were prepared from ODS rats by feeding AsA-free diet; this deficiency was confirmed by measuring the AsA level in plasma and liver homogenate. The plasma and liver AsA levels in AsA-deficient ODS rats were approximately one-thirtieth of those in AsA-supplemented rats (Table 2). In AsA-deficient rats, the blood glucose level of the STZ-treated group was 2.2 times higher compared with that of the vehicle-treated group. The increment of blood glucose level between groups treated with and without STZ in AsA-deficient rats was quite similar to that in AsA-supplemented rats. The plasma MDA

level was slightly increased by treatment of STZ in AsA-deficient rats, but the level was not significantly different in liver. These results indicate that AsA deficiency has little effect on the progress of diabetes induced by STZ.

Interestingly, the depletion of AsA in ODS rats caused a significant increase of the signal decay rate at the upper abdomen of AMC-PROXYL in STZ-treated rats (Fig. 3). The administration of allopurinol, a xanthine oxidase inhibitor, 2 h before *in vivo* EPR measurements, restored nearly 76% of the enhanced decay rate, although the difference was not significant. These results suggest that ROS generated through xanthine oxidase in intracellular sites are involved in part in the enhanced signal decay in STZ-treated rats and that the enhancement is detectable only in AsA-depleted rats.

DISCUSSION

In previous papers, the signal decay of carbamoyl-PROXYL injected intravenously was enhanced in STZ-treated rats compared with vehicle-treated rats, and the enhanced signal decay was suppressed to the control level by pretreatment with insulin or α-tocopherol and simultaneous injection of superoxide dismutase and tiron (16, 26), indicating that the enhanced signal decay was due to *in vivo* ROS generation in the hyperglycemic condition. In the present paper, the location where the enhanced signal decay of nitroxyl probes originated was determined in rats with STZ-induced diabetes using several nitroxyl probes. The partition coefficients between *n*-octanol and phosphate-buffered saline indicate a lesser membrane permeability of carbamoyl-PROXYL, no permeability of carboxy-PROXYL, and AMC-PROXYL having high permeability and retentivity in cells.

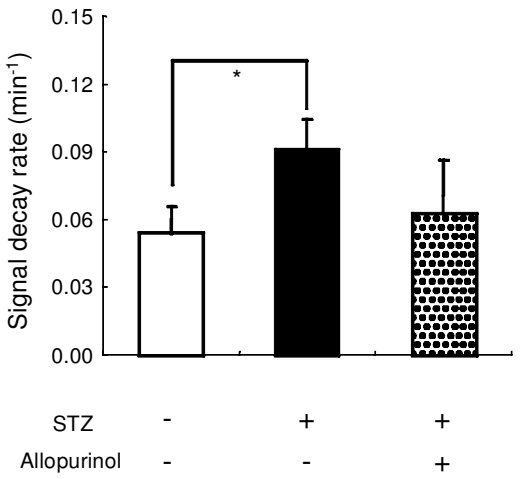

FIG. 3. The enhanced signal decay of AMC-PROXYL and the effect of allopurinol on the signal decay rate in AsA-deficient diabetic ODS rats. AMC-PROXYL was used as the spin probe for assessing the intracellular free radical reaction. Open bar is vehicle-treated group. Closed bar is STZ-treated group. Dotted bar is allopurinol-treated group. Values are mean ± SD ($n = 4–5$). *$p < 0.05$, statistically significant difference compared with control rats.

Both carbamoyl- and carboxy-PROXYLs demonstrated enhanced signal decay in STZ-treated rats compared with control rats, but AMC-PROXYL showed no difference between STZ- and vehicle-treated groups in AsA-supplemented ODS rats. AMC-PROXYL had the highest partition coefficient of the three nitroxyl probes, having membrane permeability. These results suggested that the enhanced signal decay rate occurred mainly in the intravascular site but not intracellularly in STZ-induced diabetes of AsA-supplemented ODS rats.

Recently, we reported that the enhanced plasma xanthine oxidase activity was responsible for the superoxide generation in STZ-induced diabetes (16). The increased plasma xanthine oxidase activity is reportedly due to the increased release of xanthine oxidase from liver in STZ-induced diabetes (4, 11). The overall increase in the amount of xanthine and xanthine dehydrogenase in animals with diabetes should be responsible for the increased plasma free radical reactions in the intravascular region of rats with STZ-induced diabetes. It should, however, be mentioned that other possible mechanisms could have caused the enhanced signal decay (10, 16). It is still unclear whether the enhancement of signal decay is the cause or the effect of STZ-induced diabetes.

The induction of diabetes by STZ did not increase the liver MDA level in AsA-supplemented ODS rats, although a significant increment of plasma MDA was observed. A similar tendency was demonstrated in AsA-deficient rats. The blood glucose level was significantly induced by STZ treatment without any difference between AsA-supplemented and -deficient rats. The AsA level was maintained at the control value in AsA-supplemented ODS rats by giving AsA-containing tap water. Administration of exogenous AsA to normal rats was reported to relieve the symptom of diabetes (2, 3, 9) and restore the liver MDA level to the control value (2). These facts indicate that endogenous AsA might play a different role from exogenous AsA in the development of diabetes.

The interesting finding in this study was the observation of the enhanced signal decay of AMC-PROXYL in STZ-treated rats after AsA depletion (Fig. 3). However, the liver MDA level in AsA-depleted rats did not increase compared with that in vehicle-treated rats (Table 2). Therefore, the intracellular enhancement of free radical reactions may not relate to lipid peroxidation in STZ-induced diabetes of AsA-depleted rats. The factors associated with the enhanced signal decay could involve the changes of free radical reactions, redox status, and other metabolism, and the enhancement of free radical reactions in rats with STZ-induced diabetes was suppressed by allopurinol (16, 26).

The enhanced signal decay of AMC-PROXYL caused by STZ in AsA-deficient rats tended to be suppressed by administration of allopurinol, indicating the involvement of xanthine oxidase. Allopurinol inhibits xanthine oxidase by acting at the enzyme's molybdenum center (15). The enhanced signal decay observed in STZ-induced diabetes of AsA-depleted ODS rats might be caused through electron reduction by xanthine oxidase at the intracellular region. However, the possibility of the change of redox status in an intracellular site should not be neglected. Further investigation will be needed to clarify the mechanism of enhanced nitroxyl radical decay in AsA-deficient diabetes.

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ABBREVIATIONS

AMC, 3-acetoxymethoxy; AsA, ascorbic acid; EPR, electron paramagnetic resonance; MDA, malondialdehyde; ODS, osteogenic disorder shionogi; PROXYL, 2,2,5,5-tetramethyl-1-pyrrolidinyloxy; ROS, reactive oxygen species; STZ, streptozotocin.

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Address reprint requests to:
Hideo Utsumi, Ph.D.
Laboratory of Bio-function Science
Graduate School of Pharmaceutical Sciences
Kyushu University
3-1-1 Higashi-ku
Maidashi, Fukuoka, 812-8582, Japan

E-mail: utsumi@pch.phar.kyushu-u.ac.jp

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