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Forum News & Views

Are Free Radical Reactions Increased in the Diabetic Eye?

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ABSTRACT

Reactive oxygen species (ROS) are thought to play a significant role in the development of diabetic retinopathy; however, no direct evidence supports ROS generation *in vivo*. This study used *in vivo* electron spin resonance (ESR) spectroscopy with a surface resonator to detect local free radical reactions. The ESR signal decay of carbamoyl-PROXYL was enhanced in the eyes of streptozotocin (STZ)-induced diabetic mice. This enhanced signal decay was suppressed by the administration of SOD or the pretreatment with aminoguanidine. We demonstrate, for the first time, specific free radical reactions in the eyes of mice with STZ-induced diabetes. *Antioxid. Redox Signal.* 9, 367–373.

OXIDATIVE STRESS IN DIABETIC RETINOPATHY

CHRONIC HYPERGLYCEMIA is the major determinant of diabetic retinopathy. Cell damage caused by reactive oxygen species (ROS) is believed to play a significant role in the development of diabetic retinopathy (4). Consistent with this idea, the administration of antioxidants inhibits the development of retinopathy in diabetic rats (9, 17). The treatment of diabetic animals with aminoguanidine, aspirin, or vitamin E significantly inhibits the diabetes-induced increase in superoxide anion radical (O_2^-) production in retinal tissues and cells (4). Many reports have shown enhanced oxidative stress in diabetic retinopathy; however, it has not been proved directly in live animals.

IN VIVO ESR SPECTROSCOPY/SPIN PROBE TECHNIQUE

Nitroxyl radicals are used as spin probes in a variety of biologic experiments in which electron spin resonance (ESR) spectroscopy is used to detect ROS (13, 20–22, 28) and redox status (11). Nitroxyl radicals react with O_2^- in the presence of reducing agents (10, 19) and with hydroxy radical (•OH) (18). We proposed using nitroxyl radicals as spin probes for *in vivo* ESR spectroscopy to determine the level of ROS generation under conditions of streptozotocin (STZ)-induced diabetes mellitus (13, 20–22). In these studies, the administration of antioxidants, such as superoxide dismutase (SOD) (13, 20, 22), xanthine oxidase inhibitor (13), and NAD(P)H oxidase inhibitor (22), suppressed the enhanced signal decay. The results obtained using the *in vivo* ESR/spin-probe technique showed directly the generation of ROS in mice and rats with STZ-induced diabetes. In these studies, the animal was placed in the volume-type resonator of the ESR spectrometer to detect ROS generation in the abdomen. However, the volume-type resonator is not suitable for measuring radicals in the eyes of experimental animals, because it can detect only radicals distributed throughout a larger portion of the animal's body. Instead, a surface-coil–type resonator (surface resonator) is used to measure radicals in more restricted volumes (12, 23–25).

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IN VIVO ESR WITH A SURFACE RESONATOR FOR DETECTION OF FREE RADICALS IN THE EYES OF LIVING MICE

To detect the free radical reactions in the eyes of living mice, we designed a surface resonator specifically for the size of the whole mouse eye. Figure 1A shows the experimental arrangement of the *in vivo* ESR spectroscopy. The mouse eye was located in the space bounded by a coil. The sensitivity was highest along the inside edge of the single-turn coil (Fig. 1B). The sensitivity decreased exponentially with increasing distance from the plane of the coil and normal to it, at any position relative to the coil.

Anesthetized mice were given 3-carbamoyl-2,2,5,5-tetramethylpyrrolindin-*N*-oxyl (carbamoyl-PROXYL) intravenously, and the ESR spectrum was measured noninvasively on the surface of the eye with the surface resonator. A typical ESR signal for carbamoyl-PROXYL of triplet lines was observed (Fig. 2A). The sensitivity map of the surface resonator indicated that the ESR signal detected with the surface resonator arose mainly from carbamoyl-PROXYL located adjacent to the whole eye. The intensity of the *in vivo* signal increased for up to 1–2 min after the injection and then gradually decreased (Fig. 2B). The decrease in the ESR signal after 2 min obeyed first-order kinetics. Conversely, when a loop-gap resonator was used for detecting the ESR signal of carbamoyl-PROXYL at head region including eyes, the maximum signal intensity was observed within 30 sec after injection (data not shown), which agreed with our previous report (15). The decay rate of the carbamoyl-PROXYL signal measured with the surface resonator was 0.07 ± 0.01 per min, and that of the probe measured with the loop-gap resonator was 0.10 ± 0.01 per min. These differences between two resonators were also confirmed by Takeshita *et al*. (25), which demonstrated that the ESR signal detected with the surface resonator was completely different

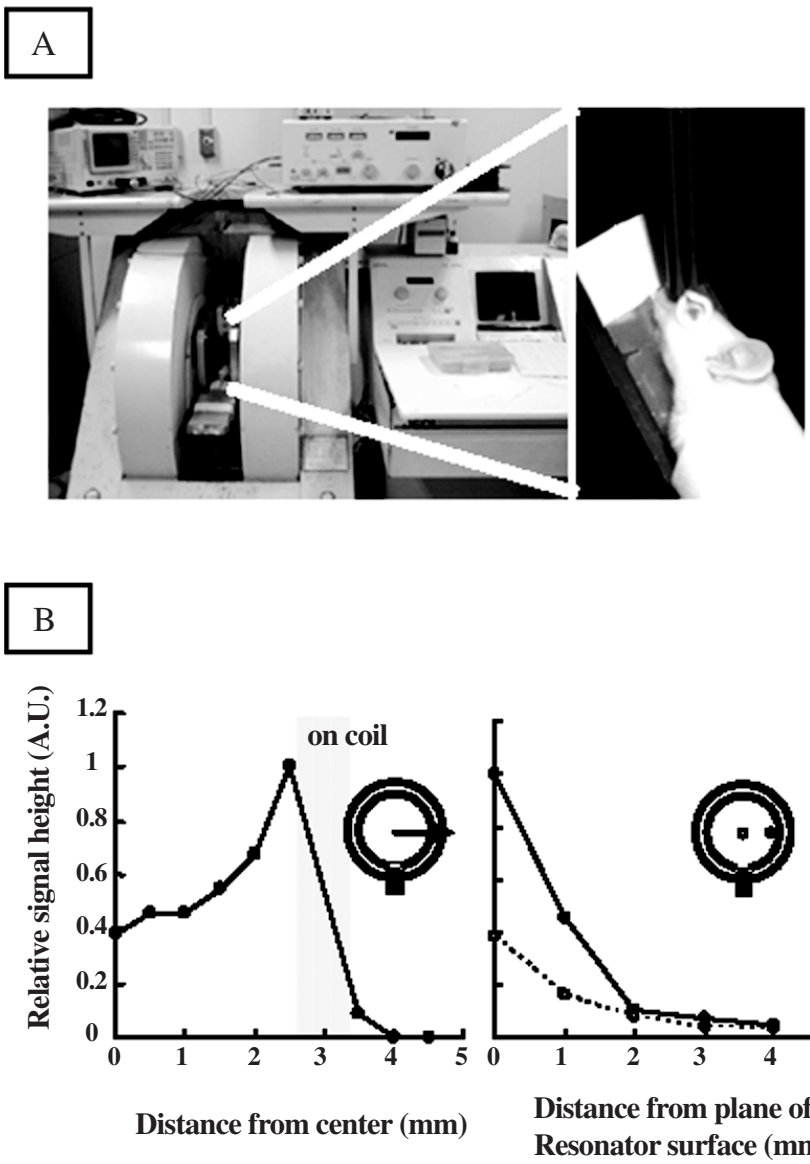

FIG. 1. L-band ESR spectrometer with a surface resonator (A) and the sensitivity distribution of the surface resonator (B). A small particle of a stable radical, 1-diphenyl-2-picrylhydrazyl radical, was placed in various positions in the plane of a single-turn loop of the surface resonator or along lines perpendicular to the resonator surface, and the relative electron spin resonance signals were measured.

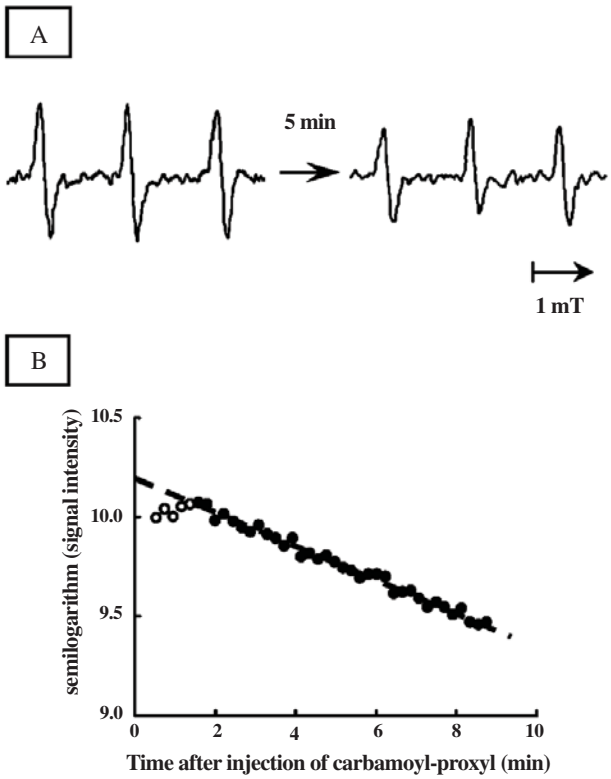

FIG. 2. Typical ESR spectrum of carbamoyl-PROXYL in the eye of a mouse (A) and the signal decay curve (B). A solution of carbamoyl-PROXYL was injected into the tail vein of the mouse.

from that of the systemic circulation and arose mainly from carbamoyl-PROXYL distributed in the skin of mouse after intravenous injection. These facts strongly suggested that surface resonator allows detection of the localized free radical.

DETECTION OF THE FREE RADICAL REACTIONS IN THE EYE OF MICE WITH STZ-INDUCED DIABETES

The body weight, glucose concentration, creatinine concentration, and fructosamine concentration in the plasma of STZ- and vehicle-treated mice are shown in Table 1. The blood glucose in the diabetic mice was approximately twice that in the vehicle-treated group after STZ treatment. The yield of colored formazan dye, which was calibrated using a fructosamine standard curve, was significantly higher than that in vehicle-treated mice 4 weeks after STZ treatment.

To provide direct evidence that the eyes of STZ-treated mice produce more ROS *in vivo* than the eyes of control mice, the *in vivo* ESR/spin-probe technique was applied to STZ- and vehicle-treated mice. A semilogarithmic plot of the signal intensity versus time was almost linear from 2 to 7 min after injection, allowing calculation of the initial velocity as the signal-decay rate (Fig. 3). The signal decay was clearly faster in the STZ-treated mouse than in the vehicle-treated mouse at 4 weeks after STZ treatment (Fig. 3A). The signal decay of the upper abdomen was enhanced at 2 weeks after STZ treatment in previous reports (13, 20, 22), but no detectable change in the eye was found at this earlier time point.

The previous results indicated that ROS generation, monitored with the *in vivo* ESR/spin-probe technique, was enhanced in STZ-treated mice and rats and that the enhanced ROS generation was related to hyperglycemia (13, 20, 22). In the present experiments, the simultaneous injection of SOD with carbamoyl-PROXYL completely suppressed the enhanced signal decay in the STZ-treated mice (Fig. 3B). These results suggest that enhanced free radical reactions were responsible for the enhanced signal decay observed in the eyes of STZ-treated mice. Peroxynitrite may also contribute to the enhanced signal decay, because SOD also inhibits peroxynitrite generation caused by the disproportion of O_2^-.

NONENZYMATIC GLYCATION REACTION AS SOURCE OF ROS

Nonenzymatic glycation is a reaction between glucose and an amino group in a protein that results in the creation of a ketoamine, and it leads to the formation of Amadori products such as fructosamine. Amadori products and derived reactive carbonyl compounds are known to generate oxygen free radicals (16). Although the glycation reaction is reversible *in vitro* and *in vivo* under normoglycemia resulting from improved glycemic control, oxygen free radicals accelerate the formation of irreversible and highly reactive adducts, the so-called advanced glycosylation end products (AGEs), and

TABLE 1. GENERAL CHARACTERISTICS OF MICE WITH STZ-INDUCED DIABETES

	2 week		*4 week*	
	Control	*Diabetic mice*	*Control*	*Diabetic mice*
Body weight (g)	38.6 ± 0.5	37.2 ± 0.7	43.8 ± 1.2	33.9 ± 1.7[b]
Glucose (mg/dl)	182.7 ± 7.8	425.3 ± 59.4[a]	196.9 ± 9.9	442.8 ± 31.1[b]
Fructosamine (μ*M*)	499.4 ± 34.0	617.2 ± 42.6	505.6 ± 18.7	666.8 ± 55.7[c]
Creatinine (mg/dl)	0.82 ± 0.09	0.87 ± 0.09	0.88 ± 0.05	0.84 ± 0.05

Each value represents the mean ± SEM.
[a]$p < 0.005$ *vs*. the 2-weenondiabetic group.
[b]$p < 0.005$ and [c]$p < 0.05$ *vs*. the nondiabetic group at 4 weeks after the injection. Eight animals were used for each experiment.

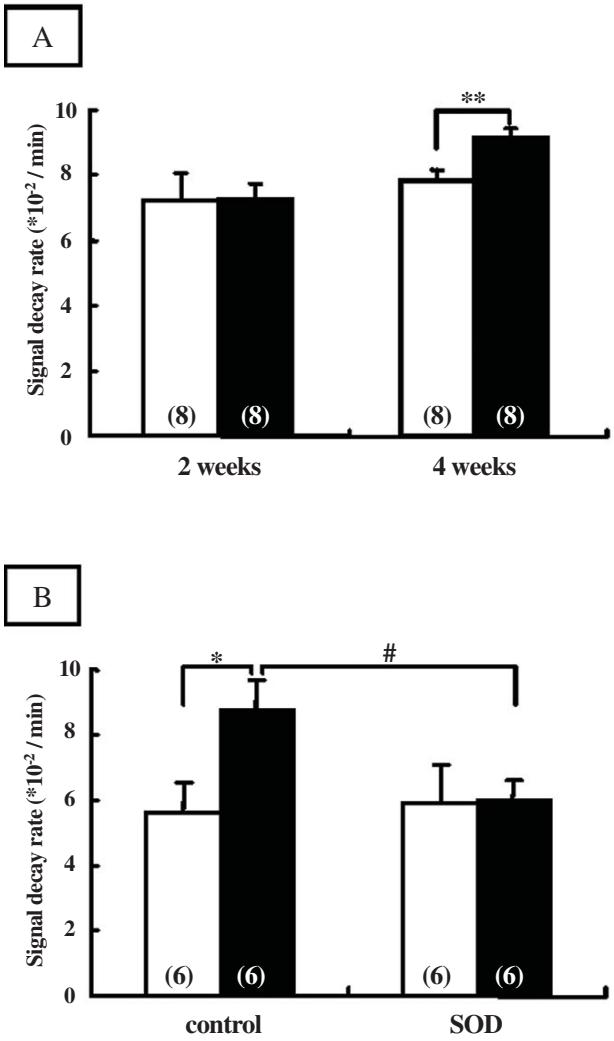

FIG. 3. Changes in the signal-decay rate in mice with STZ-induced diabetes compared with nondiabetic control mice (A) and the effect of SOD on the signal-decay rate 4 weeks after STZ treatment (B). Two and 4 weeks after the onset of diabetes, *in vivo* ESR measurements were performed. The carbamoyl-PROXYL signal-decay curve was obtained by plotting the peak height of the ESR signals semilogarithmically. The initial kinetic constant (signal-decay rate) was calculated from the slope of the signal-decay curve. *Open columns* indicate vehicle-treated mice, and *solid columns* indicate STZ-treated mice. The values in parenthesis are the numbers of animals. Each value represents the mean ± SEM; $*p < 0.05$ and $**p < 0.01$ *vs*. the nondiabetic group; and $^{\#}p < 0.05$ vs. the diabetic group treated with saline instead of SOD.

cause cross-links in protein. AGE formation is hypothesized to function in the development of diabetic cataract, and therefore pharmacologic agents have been sought to inhibit this process by blocking the glycation cascade. The nucleophilic hydrazine compound aminoguanidine is the most extensively investigated compound of this type and effectively inhibits AGE formation. Thus, combining the *in vivo* ESR/spin-probe technique with aminoguanidine should reveal the relation between glycation and the formation of *in vivo* free radical reactions in the STZ-induced diabetic cataract.

To confirm the contribution of the nonenzymatic glycation cascade to the increased free radical reaction, the mice were treated with aminoguanidine. The aminoguanidine distinctly suppressed the enhanced signal decay (Fig. 4A) in the STZ-treated mice. Plasma collected 4 weeks after STZ treatment yielded colored formazan dye from NBT (Fig. 4B). Formazan dye is generated by reactive species such as O_2^- (8) and Amadori products (7). Aminoguanidine reacts with reactive carbonyl groups, resulting in decreased NBT reduction (1). Some correlation was observed between the enhanced signal decay

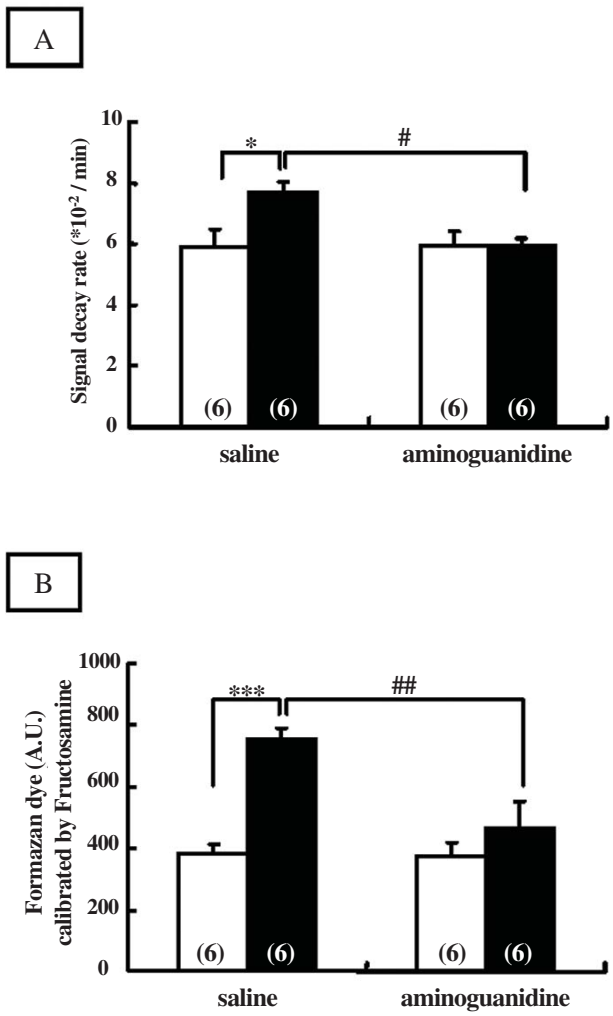

FIG. 4. Effect of aminoguanidine on the *in vivo* free radical reaction (A) and the yield of colored formazan dye (B). Aminoguanidine (100 mg/kg body weight) was intraperitoneally injected once daily on each Monday through Friday from 2 to 4 weeks after the onset of diabetes. *Open columns* indicate vehicle-treated mice, and *solid columns*. STZ-treated mice. The values in parenthesis are the numbers of animals. Each value represents the mean ± SEM; $*p < 0.05$ and $***p < 0.005$ *vs*. the nondiabetic group; and $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ *vs*. the diabetic group treated with saline instead of aminoguanidine.

and the yield of colored formazan dye ($R = 0.606$; $p < 0.026$; $n = 12$). These findings suggest that aminoguanidine inhibits free radical reactions via the nonenzymatic glycation cascade.

CONCLUSIONS AND OPEN QUESTIONS

This study detected free radical reactions in the whole eyes of living mice and confirmed that free radical reactions occur in the eyes of STZ-treated mice. To perform the experiments, we designed a surface resonator specifically for the size of the whole mouse eye. The mouse eye was located in the space bounded by a coil. We previously confirmed that the signal decay rates of carbamoyl-PROXYL varied at different regions, and the enhancement of signal decay limited to the region exposed to oxidative stress, although details are unclear (25–28). For example, in adjuvant arthritis model rats, any difference was not observed in the signal-decay rate between arthritis model and control group at the breast and abdomen, although significant enhancement was observed at the paw (27). When we used carbamoyl-PROXYL as a spin probe, the probe allowed us to detect ROS generation not in systemic circulation but at the interface of the cell membrane (28). We also confirmed that the surface resonator was sensitive only to the plane of the coil, and the sensitivity of the surface resonator was limited to the signal of carbamoyl-PROXYL distributed to the eye.

The ESR signal decay of carbamoyl-PROXYL in the eye was enhanced in mice with STZ-induced diabetes. We previously proposed using nitroxyl radicals as spin probes for *in vivo* ESR spectroscopy to determine the level of ROS generation under conditions of STZ-induced diabetes mellitus (13, 20). In these studies, the signal decay at the upper abdomen was enhanced at 2 weeks after STZ treatment, and the enhanced signal decay was related to hyperglycemia. However, in the present study, no detectable change of signal decay was found in the eye at this earlier time point, although the concentration of blood glucose in the diabetic group was higher than that of control. Interestingly, the signal decay in the eye enhanced at 4 weeks after STZ treatment when the formazan dye calibrated by fructosamine was increased. These results suggested that mechanisms of oxidative stress in the eye may be different from those of in the upper abdomen.

The enhanced signal decay was suppressed by the administration of SOD, indicating that increased free radical reactions largely accounted for the enhanced signal decay. This observation was similar to previous results demonstrating enhanced signal decay in the abdomen (13, 20–22). The reaction of carbamoyl-PROXYL with O_2^- likely occurs mainly within the lumen of capillaries in the eye, because membrane-impermeable reagents such as SOD completely inhibited the enhanced signal decay of the probe. Various mechanisms have been postulated for intravascular O_2^- generation (13, 22).

The glycation of biologic substances seems to contribute to ROS generation. Mullarkey *et al*. (16) confirmed that O_2^- was generated by the glycation of proteins, using the ESR/spin-trap technique. A diabetes-like glucose concentration increases the O_2^- production in retinal cells, and the O_2^- contributes to impaired viability and increased cell death under these circumstances (4).

The colored formazan dye is generated by the reduction of NBT by ketoamines in alkaline solution (1, 2, 7). The nucleophilic aminoguanidine can react with a reactive carbonyl groups (6), lowering the amount of NBT reduction. Aminoguanidine does not change the concentration of hemoglobin A1c, which is determined by a relatively direct method such as high-performance liquid chromatography (HPLC) or immunochemistry (3). The carbonyl structures of Amadori products may contribute to the *in vivo* free radical reactions that we confirmed using the *in vivo* ESR/spin-probe technique (Fig. 4). Hence, the reaction between aminoguanidine and the reactive carbonyls of glycation adducts may be one of the mechanisms for the inhibitory action of aminoguanidine on the free radical reactions and subsequent AGE formation. Conversely, aminoguanidine also inhibits inducible nitric oxide synthase (14) and scavenges peroxynitrite (29), so the generation of RNS in the eye might contribute to the *in vivo* free radical reactions.

We used a surface-type ESR resonator for the *in vivo* detection of free radical reactions in the whole eye. This is the first report of the *in vivo* detection of free radical reactions occurring in the eye of a living animal. *In vivo* ESR spectroscopy is likely to be useful in retinopathy, not only for studying the mechanism of injury through free radical reactions but also for screening drugs such as aminoguanidine.

ABBREVIATIONS

AGE, advanced glycosylation end product; carbamoyl-PROXYL, 3-carbamoyl-2,2,5,5-tetramethylpyrrolindin-*N*-oxyl; ESR, electron spin resonance; NBT, nitroblue tetrazolium; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; surface resonator, surface-coil-type resonator.

APPENDIX

Notes

1. Chemicals

Streptozotocin (STZ), the creatinine assay kit, the glucose assay kit, and uricase were purchased from Wako Pure Chemical Industries (Osaka, Japan). Aminoguanidine, nitroblue tetrazolium (NBT), and SOD were purchased from Sigma-Aldrich Co. (St. Louis, MO), and 3-carbamoyl-2,2,5,5-tetramethylpyrrolidine-*N*-oxyl (carbamoyl-PROXYL) was from Aldrich Chemical Co. (Milwaukee, WI). The fructosamine standard was purchased from Roche Diagnostics Japan. All other chemicals were of the highest grade.

2. Animals

Female ICR mice (age 4 weeks) were obtained from Kyudo Co., Ltd. (Fukuoka, Japan) and were acclimated for 1 week before the experiments. Diet (MF; Oriental Yeast Co. Tokyo, Japan) and water were provided *ad libitum*. At 5 weeks of age, 80 mg/kg body weight of STZ in saline was injected into the tail vein of the mice after overnight fasting, as described previously (13). Vehicle-treated animals received only saline. The mice were used for the following experiments 2 or 4 weeks after the onset of diabetes. 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 Ani-

mal Experiments of the Graduate School of Pharmaceutical Sciences, Kyushu University.

3. *In vivo* ESR measurement

An electrically tunable surface resonator consisting of a single-turn coil (6 mm in diameter), a parallel semirigid coaxial line, and matching and tuning circuits (5) was connected to an L-band ESR spectrometer (JES RE-3L; JEOL, Akishima, Japan), and a handmade 100-kHz field modulation coil was attached to the surface of the ESR magnet.

Mice were anesthetized with intraperitoneal injection of pentobarbital and placed in a handmade holder. An isotonic solution containing 150 m*M* carbamoyl-PROXYL (2.4 mmol/kg body weight) as the spin probe was injected into the tail vein, and a surface resonator was then gently attached to the eye to be examined, and ESR spectra were recorded at 12-s intervals for 10 min.

SOD (10 units/mouse) was administered simultaneously with the carbamoyl-PROXYL. Aminoguanidine (100 mg/kg body weight) was intraperitoneally injected once daily, Monday through Friday, from 2 to 4 weeks after the onset of diabetes.

4. Plasma glucose, uric acid, creatinine, and formazan dye assays

Blood was collected, heparinized, and then spun for 10 min at 900 *g*. The plasma glucose, uric acid, and creatinine concentrations were determined using an assay kit for each. The change from NBT to formazan was used as an assay to evaluate Amadori products such as fructosamine (7). Plasma was added to carbonate buffer at pH 10.3 containing NBT (0.54 m*M*) in the presence of uricase (3.78 units/ml). The absorbance at 546 nm was measured 10 and 15 min after mixing and compared with a fructosamine standard (412 μ*M*). The whole assay was carried out at 37°C.

5. Statistical analysis

Each value represents the mean ± SEM. Significant differences were determined by one-way analysis of variance (ANOVA) using the Tukey *post hoc* test. Pearson's coefficient test was used to determine correlations. Values of $p < 0.05$ were accepted as statistically significant.

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