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Confirmation of Superoxide Generation via Xanthine Oxidase in Streptozotocin-induced Diabetic Mice

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Reactive oxygen species (ROS) may play key roles in vascular inflammation and atherogenesis in patients with diabetes. In this study, xanthine oxidase (XO) system was examined as a potential source of superoxide in mice with streptozotocin (STZ)-induced experimental diabetes. Plasma XO activity increased 3-fold in diabetic mice ($50 \pm 33\,\mu U/ml$) 2 weeks after the onset of diabetes, as compared with non-diabetic control mice ($15 \pm 6\,\mu U/ml$). *In vivo* superoxide generation in diabetic mice was evaluated by an *in vivo* electron spin resonance (ESR)/spin probe method. Superoxide generation was significantly enhanced in diabetic mice, and the enhancement was restored by the administration of superoxide dismutase (SOD) and 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron), which was reported to scavenge superoxide. Pretreatment of diabetic mice with XO inhibitors, allopurinol and its active metabolite oxipurinol, normalized the increased superoxide generation. In addition, there was a correlation ($r = 0.78$) between the level of plasma XO activity and the relative degree of superoxide generation in diabetic and non-diabetic mice. Hence, the results of this study strongly suggest that superoxide should be generated through the increased XO seen in the diabetic model mice, which may be involved in the pathogenesis of diabetic vascular complications.

Keywords: Diabetes; Electron spin resonance; Nitroxyl radical; Streptozotocin; Superoxide; Xanthine oxidase

Abbreviations: Carbamoyl-PROXYL, 3-Carbamoyl-2,2,5,5-tetramethylpyrrolindin-1-yloxy; ESR, electron spin resonance; ROS, reactive oxygen species; SOD, superoxide dismutase; STZ, streptozotocin; XDH, xanthine dehydrogenase; XO, xanthine oxidase

INTRODUCTION

Vascular diseases, including atherosclerosis and micro-angiopathy, are the principal causes of mortality and morbidity in patients with diabetes mellitus.[1,2] Numerous studies have demonstrated the increased generation of reactive oxygen species (ROS) in animal models and patients with diabetes. The increased ROS generation may be involved in the onset and development of vascular complications.[3–5] Various mechanisms have been postulated for ROS generation: (1) glycation of biological substances, (2) alteration of the polyol pathway, (3) activation of NAD(P)H oxidase, and (4) through activated endothelial nitric oxide synthase.[6–8]

In addition to these systems, xanthine oxidase (XO) system seems to contribute to ROS generation. Butler *et al.*[9] reported that the oral administration of allopurinol, which is a XO inhibitor, normalizes endothelial dysfunction in Type 2 diabetics, suggesting the involvement of ROS generation through xanthine/XO system in diabetic vascular complications. Moreover, Cosic *et al.*[10] demonstrated the elevation of circulating XO activity in patients with Type 2 diabetes. Quite recently, Desco *et al.*[11] reported that streptozotocin (STZ) caused a significant increase in both hepatic XO and XDH activities and the release of XO from liver in rats. They also demonstrated the enhancement of lipid peroxidation in diabetic rats, which was diminished

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with allopurinol, and suggested that XO is responsible for ROS generation. All these reports suggest the possibility of XO as a source of ROS in diabetes, however there is no direct evidence for superoxide anion radical generation.

Although there are some discrepant reports showing the different origin of ROS generation, the determination of the source of ROS should be most important to clarify the molecular mechanism of diabetes and to find good care for the disease. In order to clarify the mechanism of diabetic complications, *in vivo* observation of ROS generation using disease models should be very useful and promising. Both of drug-induced and spontaneous models are widely used as animal model for diabetes. Increased ROS generation, including superoxide and hydroxyl radicals, has been well characterized in the animal model of STZ-induced diabetes.[12,13] In fact, a significant increase of XO activity was demonstrated in both liver and kidney in STZ-treated rats.[11,14]

To non-invasively evaluate ROS generation *in vivo*, electron spin resonance (ESR)/spin probe method should be a powerful technique. We succeeded to confirm the increased ROS generation in animal models with iron-overload[15] or diesel exhaust particles.[16] In diabetic model, we demonstrated a significant increase of ROS generation in STZ-induced diabetic rats.[17]

In the present study, we confirmed that STZ caused an significant increase in both plasma XO and XDH activities in mice and that the increase of XO activity is responsible for *in vivo* superoxide generation using non-invasive ESR/spin probe method.

MATERIALS AND METHODS

Chemicals

STZ and 4,5-dihydroxy-1,3-benzene disulfonic acid (Tiron) were purchased from Sigma-Aldrich Co. (St Louis, MO, USA), and 3-carbamoyl-2,2,5,5-tetramethylpyrrolindin-1-yloxy (carbamoyl-PROXYL) was from Aldrich Chemical Co. (Milwaukee, WI, USA). Pterin, allopurinol, oxipurinol, superoxide dismutase (SOD) and uric acid assay kit were from Wako Pure Chemical Industries (Osaka, Japan). All other chemicals were of the highest grade.

Animals

Female ICR mice (age 3 weeks) were obtained from Seac Yoshitomi Co. (Fukuoka, Japan), and were acclimatized for 1 week before the experiments. Diet (MF, Oriental Yeast Co. Tokyo, Japan) and water were provided *ad libitum*. At 4 weeks of age, 100 mg/kg body weight of STZ in saline was injected into the tail vein of the mice after an overnight fast. Vehicle-treated animals received only saline. Two weeks after the treatment of STZ, blood glucose was examined and mice with a blood glucose exceeding 300 mg/dl were used as diabetic models to the following experiments. All procedures and animal care were performed in accordance with the Japanese Government Animal Production and Management Law (No. 105).

Measuring Plasma XO and Dehydrogenase Activity

Blood was collected by cardiac puncture, heparinized, and then centrifuged for 10 min at 900*g*. Plasma XO and xanthine dehydrogenase (XDH) activity were measured using the fluorometric pterin assay described by Beckman *et al*.[18] The formation of isoxanthopterin was monitored using a fluorophotometer (Fluoroskan Ascent, Thermo Labsystems, Vantaa, Finland, ex. 355 nm and em. 405 nm). The total activity of XO + XDH was determined by the addition of methylene blue.[19] One unit of enzyme activity was defined as 1 μmol of isoxanthopterin formation per minute at 37°C and pH 7.4. The specificity of the assay was verified by inhibition with allopurinol.

In Vivo ESR Measurement

Mice were anaesthetized by intraperitoneal injection of pentobarbital and fixed on a handmade holder. A 32 nmol/g body weight of carbamoyl-PROXYL solution (80 mM in phosphate buffered saline, pH 7.4) was injected into the tail vein, and then ESR spectra were measured at the upper abdominal domain using an ESR spectrometer (JES RE-3L, JEOL, Akishima, Japan) as described previously.[15,17] The microwave frequency was 1.2 GHz and the power was 1.0 mW. The amplitude of the 100-kHz field modulation was 0.125 mT.

Tiron (0.08 or 0.4 μmol/g body weight) or SOD (4 or 20 units/mouse) were simultaneously administered with carbamoyl-PROXYL. Allopurinol (40 mg/kg body weight) or oxipurinol (40 mg/kg body weight) were intraperitoneally injected 20 min before ESR measurement.

Plasma Uric Acid Measurement

The plasma uric acid concentration of vehicle- or STZ-treated mice was determined using a uric acid assay kit.[20]

Statistical Analysis

Each value represents the mean ± SD. Student's *t*-test was used to determine the significance of

TABLE I Plasma xanthine oxidase activity and uric acid concentration 2 weeks after the onset of diabetes

	Blood glucose (mg/dl)	XO (μU/ml)	Total XO+XDH (μU/ml)	Uric acid (mg/dl)
Vehicle	182 ± 19 ($n = 7$)	15 ± 6 ($n = 7$)	39 ± 29 ($n = 7$)	0.71 ± 0.12 ($n = 6$)
STZ	560 ± 70[a] ($n = 11$)	50 ± 33[a] ($n = 11$)	146 ± 62[a] ($n = 11$)	1.04 ± 0.20[b] ($n = 6$)

Each value represents the mean ± SD, and the superior a and b indicate significant difference from the vehicle-treated mice. [a]$p < 0.01$. [b]$p < 0.05$.

differences between two groups. Analysis of variance (ANOVA) followed by Fisher's PLSD was used to compare differences among more than three groups. Pearson's coefficient test was used to determine correlations. Values of p less than 0.05 were accepted as statistically significant.

RESULTS

Plasma XO Activity in STZ-induced Diabetic Mice

Table I demonstrates glucose concentration, XO activity, and total activity of XO + XDH in the plasma of STZ- and vehicle-treated mice. The blood glucose in diabetic mice was approximately 3-times higher than in the vehicle-treated group. XO activity and the total XO + XDH activity in STZ-treated mice were 3.3- and 3.7-times higher than those in the vehicle-treated group, respectively, indicating that both of XO and XDH were induced equally by STZ-treatment. The ratios of XO activity to total XO + XDH activity in the plasma of vehicle- and STZ-treated groups were 0.38 and 0.34, respectively. The similar ratio indicates that the conversion of XDH to XO was not influenced by STZ-treatment.

The concentration of plasma uric acid in STZ-treated mice was 1.5-times higher than that in vehicle-treated mice. This may suggest that the xanthine/XO system in STZ-induced diabetic mice works more than that in the control mice, causing more superoxide generation.

Enhancement of Superoxide Generation in STZ-treated Mice

In order to provide direct evidence that STZ-treated mice produce more superoxide *in vivo* than the control mice, *in vivo* ESR/spin probe technique was applied to both mice, STZ- and vehicle-treated mice. Immediately after intravenous injection of carbamoyl-PROXYL, ESR spectrum of the probe was traced at the upper abdomen of the mouse with an *in vivo* ESR spectrometer. Typical ESR signal having triplet lines shown in Fig. 1A was observed, and the signal intensity gradually decreased with time. A semilogarithmic plot of the signal intensity versus time was almost linear during the initial 5 min, allowing calculation of the initial velocity as the signal decay rate (Fig. 1B). The signal decay was clearly faster in STZ-treated mouse than that in vehicle-treated one, and was well correlated with the blood glucose level ($r = 0.85$, $n = 27$) (Fig. 4A). This observation was quite similar to the previous results obtained with an STZ-treated rat model, that the enhanced signal decay observed in the STZ-treated rats was completely suppressed with insulin therapy and antioxidants treatment, such as vitamin E.[17] The previous results strongly indicated that ROS generation monitored with *in vivo*

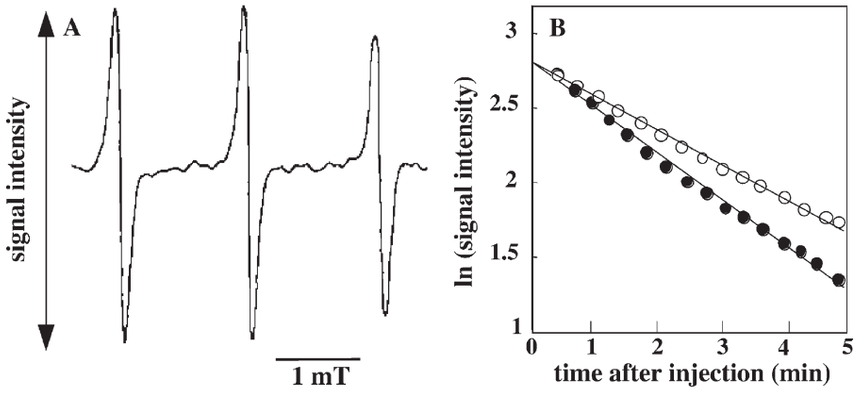

FIGURE 1 Typical ESR spectrum of carbamoyl-PROXYL in the upper abdomen domain of a mouse (A) and the signal decay curve in vehicle- and STZ-treated mice (B). Two weeks after the onset of diabetes, a solution of carbamoyl-PROXYL (80 mM, 4 μl/g body weight) was injected into the tail vein of the mice. The ESR signal intensity gradually decreased after administration. The carbamoyl-PROXYL signal decay curve was obtained by plotting the peak height of ESR signals semilogarithmically. The initial kinetic constant (signal decay rate) was calculated from the slope of the signal decay curve. Open circles indicate vehicle-treated mouse, and solid circles indicate STZ-treated mouse.

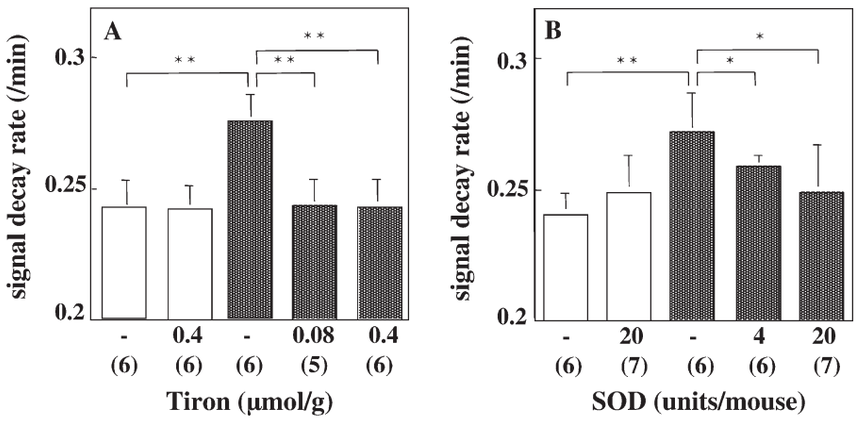

FIGURE 2 Changes in the signal decay rate in mice with STZ-induced diabetes compared with non-diabetic control mice and the effect of superoxide scavengers on the signal decay rate. Two weeks after the onset of diabetes, *in vivo* ESR measurement was performed, and the carbamoyl-PROXYL signal decay rates were obtained. (A) 0.08 or 0.4 μmol/g body weight Tiron was administrated with carbamoyl-PROXYL. (B) 4 or 20 units SOD were administrated with carbamoyl-PROXYL. Each value represents the mean ± SD. $*p < 0.05$, $**p < 0.01$. Open columns indicate vehicle-treated mice, and solid columns indicate STZ-treated mice. The values in parenthesis are the numbers of animals.

ESR/spin probe technique was enhanced in STZ-treated rats and that the enhanced ROS generation was related with hyperglycemia. In the present experiments, the simultaneous injection of Tiron, which was reported as a membrane-permeable superoxide scavenger,[21,22] with carbamoyl-PROXYL completely suppressed the enhanced signal decay in the STZ-treated mice (Fig. 2A). Administration of SOD also significantly decreased the enhanced signal decay in the STZ-treated mice (Fig. 2B). The addition of SOD and Tiron did not affect the signal decay rate in vehicle-treated mice. These results suggest that the enhanced superoxide generation is responsible for the enhanced signal decay observed in STZ-treated mice. In the other words, the enhanced signal decay in STZ-treated mice demonstrates the enhancement of superoxide generation in STZ-treated mice.

Contribution of the Xanthine/XO System to Enhanced Superoxide Generation

To confirm the contribution of the xanthine/XO system to the increased superoxide generation in STZ-induced diabetic mice, the mice were treated with the XO inhibitor allopurinol or its active metabolite oxipurinol. Both allopurinol and oxipurinol treatments significantly suppressed the enhanced signal decay in STZ-treated mice, and the suppression was not completely but about 80% to the control level. Neither allopurinol nor oxipurinol had effect on vehicle-treated mice (Fig. 3). In fact, the enhanced signal decay was observed in a few mice having low plasma XO activity, although there was some correlation between the enhanced signal decay and the plasma XO activity ($r = 0.78$, $n = 27$) (Fig. 4B). These results suggest that the xanthine/XO system mostly contributes to the excess superoxide generation observed in STZ-induced diabetic mice.

DISCUSSION

This study provides the first evidence for the excess superoxide generation through the increased XO in STZ-induced diabetic mice.

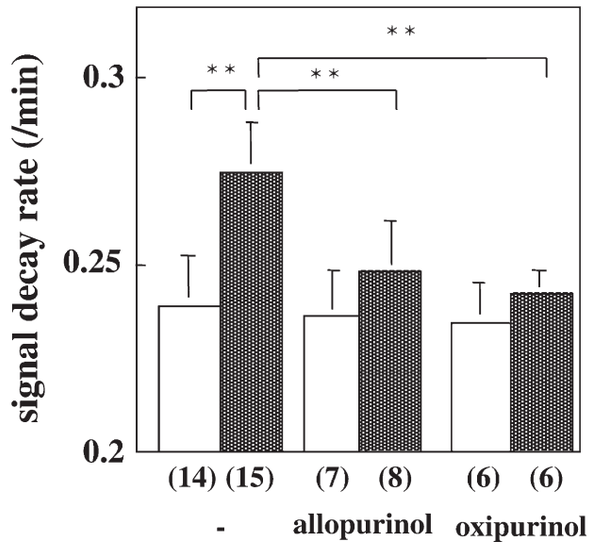

FIGURE 3 Effect of XO inhibition on the signal decay in mice with STZ-induced diabetes. Allopurinol (40 mg/kg body weight) or oxipurinol (40 mg/kg body weight) was injected intraperitoneally 20 min before ESR measurement. Each value represents the mean ± SD. $**p < 0.01$. Open columns indicate vehicle-treated mice, and solid columns indicate STZ-treated mice. The values in parenthesis are the numbers of animals.

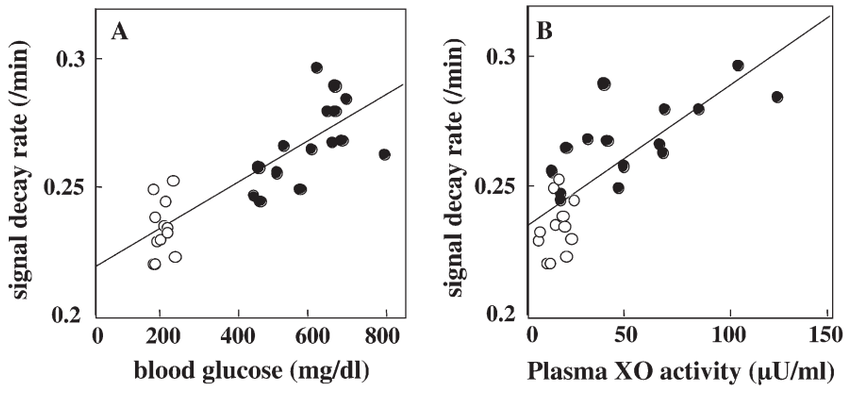

FIGURE 4 Correlations between blood glucose, plasma XO activity, and signal decay rate. (A) Correlation between blood glucose and the signal decay rate ($r = 0.85$, $n = 27$). (B) Correlation between plasma XO activity and the signal decay rate ($r = 0.78$, $n = 27$). Open circles indicate vehicle-treated mice, and solid circles indicate STZ-treated mice.

Increased XO activity has been observed in the plasma of patients with Type 2 diabetes.[10] The XO activity was also reported to be 1.5 fold higher in the plasma of STZ-treated rat than that in control one.[11] In the present study, the plasma XO activity in STZ-induced diabetic mice was 3.3-times higher than that in non-diabetic control mice. It has not been clear whether the increase of XO activity in diabetic conditions is due to XDH–XO conversion or not. The present results clearly demonstrated that the elevated plasma XO activity was not due to XDH–XO conversion, because both XO and XDH levels in blood were increased in diabetic mice and the ratios of XO activity to total XO + XDH activity of both vehicle- and STZ-treated groups were similar. An overall increase in the amounts of XO and XDH in animals with diabetes may be responsible for the increased plasma XO activity. In STZ-induced diabetic animals, XO activity also increased in liver and kidney.[11,14] Because STZ was reported to induce some liver damage,[23] it might be possible that the increase of plasma XO activity is due to nonspecific protein leakage from liver by the toxicity of STZ itself. Desco *et al.*, however, reported that XO was released from liver in STZ-induced diabetic rats without release of other hepatic enzymes such as alanine amino transferase.[11] Cosic *et al.* reported that plasma XO activity also increased in Type 2 diabetic patients.[10] Furthermore, we previously showed that the excess superoxide generation in STZ-treated rats was restored by insulin therapy.[17] These facts suggest that the increase of plasma XO activity in STZ-treated animals should be correlated with hyperglycemia, not due to hepatic toxicity of STZ itself.

In this study, we evaluated superoxide generation by measuring the enhanced signal decay with an *in vivo* ESR/spin probe method. In this method, ROS such as superoxide convert nitroxyl radicals into the corresponding non-paramagnetic compounds.[24,25] Using this method, we previously reported on hydroxyl radical generation originating from superoxide in the failing myocardium.[26,27] There was a significant correlation between the myocardial ROS level measured by ESR and left ventricular contractile dysfunction. Furthermore, we recently applied this method to measurement of free radicals *in vivo*, and succeeded in showing enhanced ROS generation in living animals with iron-overload, diesel exhaust particle-induced pulmonary injury, and STZ-induced diabetes.[15–17] Therefore, ESR/spin probe method should be a powerful and useful technique to non-invasively evaluate ROS generation *in vivo*. In the present study, the enhanced signal decay was again observed as well as the previous reports. The enhanced signal decay was completely suppressed with the simultaneous injection of Tiron, which was reported as a membrane-permeable superoxide scavenger.[21,22] Administration of SOD also significantly suppressed the enhanced signal decay in the STZ-treated mice. These results suggest that the enhancement of superoxide generation is responsible for the enhanced signal decay observed in STZ-treated mice.

There was a correlation between the enhanced signal decay and the plasma XO activity, and the treatment of mice with the XO inhibitor allopurinol or its active metabolite oxipurinol significantly suppressed the enhanced signal decay in STZ-treated mice. These results suggest that the xanthine/XO system mostly contributes to the excess superoxide generation observed in STZ-induced diabetic mice. It should, however, be noteworthy that the suppression was not completely but about 80% to the control level. The enhanced signal decay was observed in a few mice having low plasma XO activity. Therefore, it should not be neglected the possibility that the other origin than XO system takes a part in superoxide generation in some of STZ-induced diabetic mice.

In conclusion, this study confirmed the evidence for the enhancement of the superoxide generation via the increased XO in STZ-induced diabetic mice. In addition, we demonstrated that non-invasive *in vivo* ESR measurement provides useful information for evaluating endogenous ROS in diabetic conditions, and this is expected to become applicable to patients with diabetes in the near future.

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