

Superoxide dismutase reduces the impairment of endothelium-dependent relaxation in the spontaneously hypertensive rat aorta

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Abstract

The involvement of the superoxide anion in endothelium-dependent relaxation (EDR) was examined in noradrenaline-contracted aortic smooth muscle preparations isolated from normotensive Wistar Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP). Acetylcholine (ACh, 10^{-9} – 10^{-5} M) induced EDR in both WKY and SHRSP preparations in a concentration-dependent manner, but with a significantly smaller amplitude in those from SHRSP than in those from WKY. The ACh-induced EDR was inhibited by N^o-nitro-L-arginine (L-NOARG), in a concentration-dependent manner, both in WKY and SHRSP. The EDR produced in WKY in the presence of 3×10^{-6} M L-NOARG was similar in magnitude to that produced in SHRSP in the absence of L-NOARG. Superoxide dismutase (SOD, 300 units/ml) increased the amplitude of EDR in SHRSP but not in WKY, with no alteration of the threshold or of the maximal amplitude. The maximal amplitude of EDR produced in SHRSP in the presence of SOD was still smaller than that in WKY. In WKY, a possible involvement of superoxide in the EDR was examined in aortae whose EDR was partially inhibited by treatment with a subthreshold concentration (3×10^{-6} M) of L-NOARG. In the L-NOARG-conditioned aorta, the reduced EDR was partially but significantly recovered by SOD. These results suggest that the impaired EDR in aortae of SHRSP may be causally related to a higher production of superoxide. The L-NOARG-induced inhibition of EDR in WKY may be produced, in part, by the reduction of effective NO due to its destruction by superoxide.

Key words: superoxide, stroke-prone spontaneously hypertensive rats (SHRSP), aorta, endothelium-dependent relaxation, nitric oxide

Introduction

Endothelium-dependent relaxation (EDR) has been reported to be impaired in arteries of spontaneously hypertensive rats (SHR) and stroke-prone SHR (SHRSP), to an extent dependent on age and degree of hypertension (Hongo *et al.*, 1988; Sunano *et al.*, 1989; Tominaga *et al.*,

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1994; Lang *et al.*, 1995; Mantelli *et al.*, 1995; Sunano *et al.*, 2000). The cause of the impairment varies between different arteries. For example, it has been shown to be due to the decreased release or response to endothelium-derived relaxing factor (EDRF) (Grunfeld *et al.*, 1995; Hirata *et al.*, 1996), to increased release of endothelium-derived contracting factor (EDCF) (Lüscher and Vanhoutte, 1986; Watt and Thuston, 1989; Diederich *et al.*, 1990) and to decreased endothelium-dependent hyperpolarization (Fujii *et al.*, 1992, 1993; Sunano *et al.*, 1999).

EDRF may be nitric oxide (NO) (Palmer *et al.*, 1987), and the production of NO from L-arginine could be blocked by N^o-nitro-L-arginine (L-NOARG) (Moore *et al.*, 1990). Since EDRs of the aortae of both normotensive Wistar Kyoto rats (WKY) and SHRSP are largely inhibited by L-NOARG, it is considered that the EDRs are produced mainly by NO (Sekiguchi *et al.*, 1996). The reduction of EDR in SHRSP aortae may be the result of a decreased release of NO or a decreased response of smooth muscle to NO. However, the evidence from several studies indicates that a decreased release of NO is the more likely cause of the reduction, since the response of vascular smooth muscle to NO is not decreased in the aortae of SHRSP (Koga *et al.*, 1989; Osugi *et al.*, 1990; Tesfamariam and Ogletree, 1995). NO is destroyed by the superoxide anion (Gryglewski *et al.*, 1986; Palmer *et al.*, 1987), and superoxide is broken down by superoxide dismutase (SOD) (Gryglewski *et al.*, 1986). Thus it is possible that the reduced EDR of aorta in these hypertensive animals could be reversed by SOD. This is supported by the evidence that EDRs in the aortae of SHR and SHRSP are improved by SOD, possibly because it prevents the destruction of NO by superoxide (Nakazono *et al.*, 1991; Grunfeld *et al.*, 1995).

The present experiments were carried out to investigate a possible involvement of superoxide in the impairment of EDR in the aortae of SHRSP, and the results were compared with those obtained in aortae of WKY treated with L-NOARG.

Materials and Methods

Animals

Rats used in the present experiment were handled according to the "Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences" of the Physiological Society of Japan.

Male SHRSP and normotensive WKY were used in the present experiments. These rats were obtained from Shimizu Laboratory Supplies Co. Ltd. (Kyoto, Japan) at the age of 4 weeks and maintained in our animal facility until they were sacrificed. The rats were killed at the age of 16 weeks by bleeding from the *anterior vena cavae* during CO₂ anesthesia. Aortae were removed from the thoracic cavity and immersed in a modified Tyrode's solution described below.

Solutions

The composition of the modified Tyrode's solution was (mM): NaCl, 137; KCl, 5.4; CaCl₂, 2.0; MgCl₂, 1.0; NaHCO₃, 11.9; NaH₂PO₄, 0.4; glucose, 5.6; equilibrated with a gas mixture of 95% O₂ and 5% CO₂. The pH of the solution at 37°C was 7.3. High-K⁺ Tyrode's solution containing 50 mM K⁺ was made by substituting NaCl in the modified Tyrode's solution for equimolar KCl.

Preparations

Ring preparations of the aortae (1.0 mm in width) were made by cutting the aorta transversely with fine surgical scissors. In one tenth of the preparations, the endothelium was removed by rubbing the inner surface of the ring preparations gently with a soft rubber band. Two tungsten wires of 30 μm diameter were inserted into the lumen of each preparation and then one of them was tied to a plastic holder. This holder was mounted in an organ bath (10 ml) filled with modified Tyrode's solution kept at 37°C. The other wire was connected to a force transducer (Minebea, Nagano, Japan), so that changes in tension (contraction and relaxation) could be observed isometrically.

Mechanical recording

Preparations were equilibrated in modified Tyrode's solution for 60 min, then initially subjected to two successive high- K^+ -induced contractions of 30 min duration with an interval of 30 min, by changing the incubation medium from normal Tyrode's to high-K Tyrode's solution. After this procedure, the preparations were contracted by 5×10^{-7} M noradrenaline (NA). Ten min after the preparation achieved contraction maximum, acetylcholine (ACh) was applied cumulatively to induce EDR. Superoxide dismutase (SOD) was applied 30 min before the initiation of the NA contraction and was present throughout both the NA-induced contraction and the ACh-induced relaxation. In the experiment where the relaxation was depressed by L-NOARG, SOD (3×10^{-6} M or 10^{-4} M) was added to the modified Tyrode's solution 30 min before the initiation of the NA-induced contraction and remained during throughout the contraction and the ACh-induced relaxation.

Drugs

Drugs used in the present experiment were noradrenaline bitartrate (Sigma, St. Louis, MO, USA), acetylcholine chloride (Wako Chem., Osaka, Japan), N^{ω} -nitro-L-arginine (Sigma, St. Louis, MO, USA) and bovine erythrocyte superoxide dismutase (SOD, Wako Chem., Osaka, Japan). Stock solutions of noradrenaline, acetylcholine, N^{ω} -nitro-L-arginine and SOD were prepared by dissolving these chemicals into distilled water at concentrations of 0.1–10 mM or 30,000 unit/ml for SOD, and they were then added to Tyrode's solution to obtain the desired concentrations. The amount of solvent added in Tyrode's solution did not exceed 1/100 of the solution. The addition of these chemicals to Tyrode's solution did not alter the pH of the solution.

Statistics

Results are expressed as the mean \pm S.E.M. with the number of observations in parenthesis. Relaxation responses are expressed as percentages of the NA-induced contraction. Differences between the preparations from WKY and SHRSP, between the values in the presence and absence of L-NOARG, and between the values in the absence and presence of SOD were analyzed using the unpaired Student's-t test, where a P value smaller than 0.05 was considered to be significant.

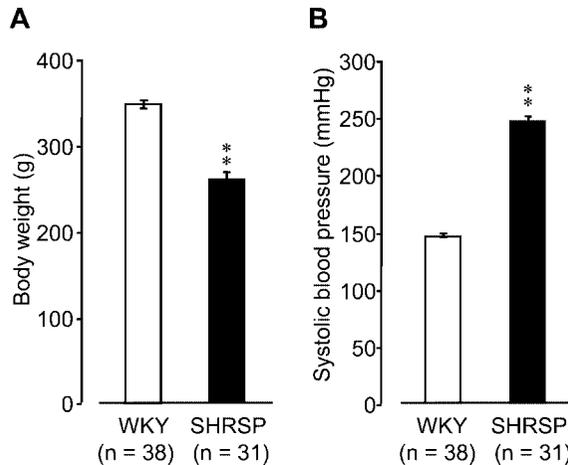


Fig. 1. Body weight (A) and systolic blood pressure (B) of Wistar Kyoto rats (WKY) and stroke-prone spontaneously hypertensive rats (SHRSP) at the age of 16 weeks. Asterisks indicate significant differences from the values of WKY (**: $P < 0.001$).

Results

Body weight and blood pressure of rats

The body weight and systolic blood pressure of WKY and SHRSP were measured just before sacrificing the animals. As shown in Fig. 1, while the systolic blood pressure was markedly higher in SHRSP compared to WKY, the opposite was the case for body weight.

Endothelium-dependent relaxation of aortae from WKY and SHRSP

In aortic rings precontracted with noradrenaline (NA, 5×10^{-7} M), acetylcholine (ACh) produced a relaxation response which was absent in tissues after removal of the endothelial cells by rubbing (*i.e.*, endothelium-dependent relaxation, EDR). The amplitude of ACh-induced EDR increased in a concentration-dependent manner, both in SHRSP and WKY (Fig. 2). For both WKY and SHRSP, the threshold concentration of ACh for the EDR was 10^{-10} M, with the maximum response at 10^{-6} M. The amplitude of the maximum relaxation was significantly ($P < 0.001$) smaller in aortae from SHRSP (about 70% of NA contraction) than in those from WKY (about 90% of NA contraction).

Experiments were carried out to test the effects of L-NOARG on the ACh-induced EDR in aortae from WKY and SHRSP. As shown in Fig. 3, the EDR was inhibited by N^o-nitro-L-arginine (L-NOARG, 3×10^{-6} – 10^{-4} M) in a concentration-dependent manner, at any given concentrations of ACh, and EDR was abolished at 10^{-4} M L-NOARG, both in WKY and SHRSP. In the presence of 3×10^{-6} M L-NOARG, the EDR for aortae from WKY was identical to that for aortae from SHRSP in the absence of L-NOARG. These results confirmed the previous observation that in both WKY and SHRSP the ACh-induced EDR was produced mainly by endothelial NO (Sekiguchi *et al.*, 1996).

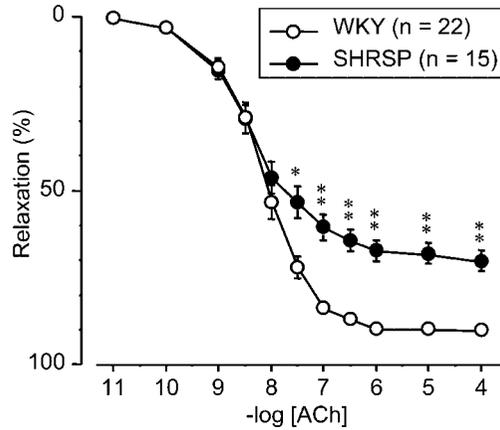


Fig. 2. Concentration-response curves for acetylcholine (ACh)-induced endothelium-dependent relaxation in aortae from WKY (open circles) and SHRSP (filled circles). Aortic rings precontracted by noradrenaline (5×10^{-7} M) were stimulated with cumulatively increasing concentrations of ACh (10^{-11} – 10^{-4} M), and the amplitude of relaxation was expressed as percentage of the contraction produced before application of ACh. Statistical significance in comparison to values in WKY was tested (*: $P < 0.05$, **: $P < 0.001$).

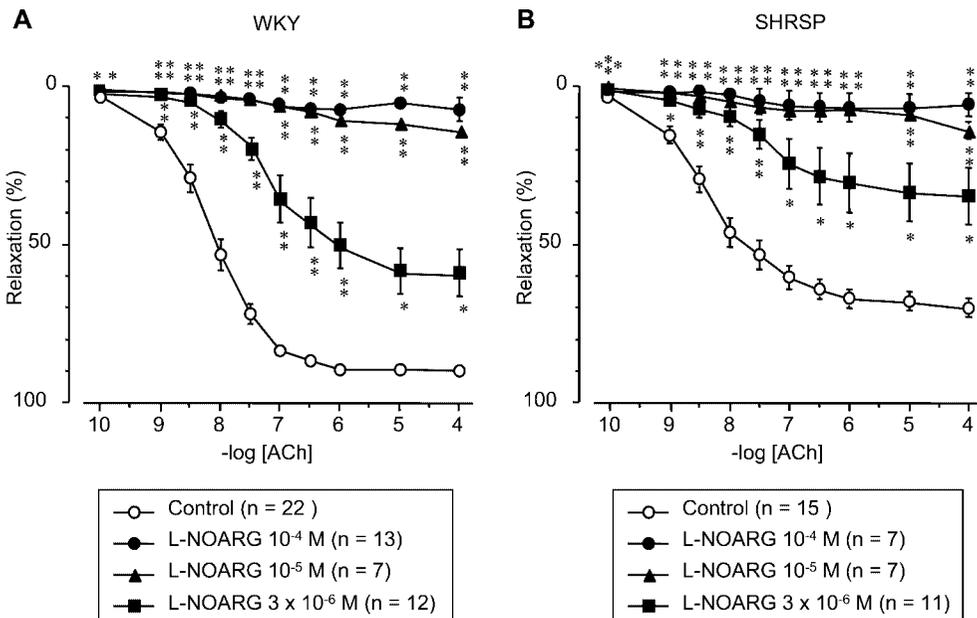


Fig. 3. Effects of N^o -nitro-L-arginine (L-NOARG) on endothelium-dependent relaxation produced by acetylcholine (ACh) in aortae obtained from WKY (A) and SHRSP (B). Aortic rings were contracted by noradrenaline (5×10^{-7} M), and cumulative concentrations of ACh (10^{-10} – 10^{-4} M) were applied in the absence (Control, open circles) and presence of different concentrations of L-NOARG (3×10^{-6} M, filled squares; 10^{-5} M, filled triangles; 10^{-4} M, filled circles). L-NOARG was applied 30 min before the initiation of noradrenaline-induced precontraction. Statistical significance compared to the values in the absence of L-NOARG (Control) was tested (*: $P < 0.05$, **: $P < 0.001$).

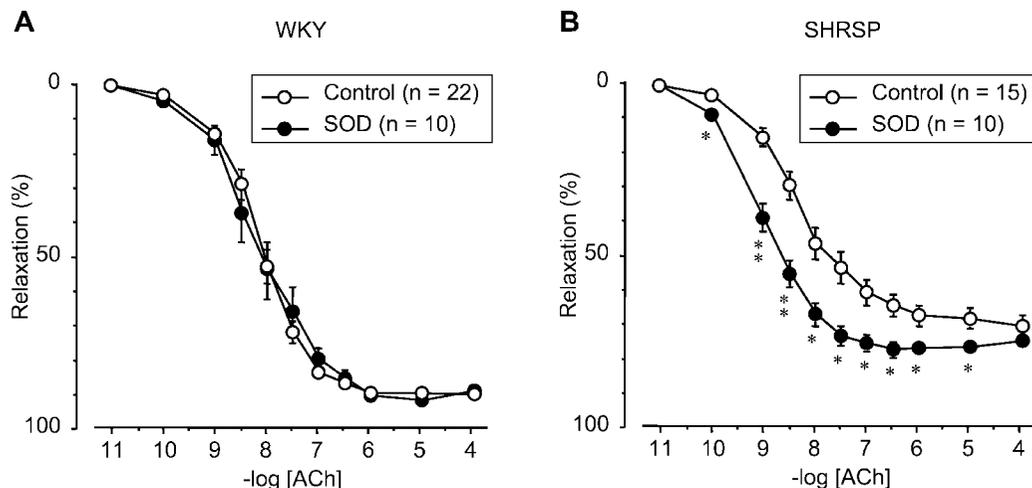


Fig. 4. Effect of superoxide dismutase (SOD) on endothelium-dependent relaxation induced by acetylcholine (ACh) in aortae from WKY (A) and SHRSP (B). Aortic rings were contracted with noradrenalline (5×10^{-7} M), and relaxations produced by cumulative application of ACh (10^{-11} – 10^{-4} M) were observed before (Control, open circles) and during the presence of SOD (300 units/ml) for over 30 min (filled circles). Statistical significance compared to Control value at each concentration of ACh was tested (*: $P < 0.05$, **: $P < 0.001$).

Effects of superoxide dismutase on endothelium-dependent relaxation

The effects of superoxide dismutase (SOD) on EDR were studied in aortic rings obtained from WKY and SHRSP. SOD (300 units/ml) did not alter the NA-induced contractions. However in the presence of SOD, the ACh-induced EDR was significantly increased in SHRSP (Fig. 4B) but no significant change was observed in WKY, at any given concentrations of ACh (Fig. 4A). The threshold concentration of ACh required for generation of EDR (equal to 10^{-10} M) was also not changed by SOD in both WKY and SHRSP.

Experiments were carried out to observe the effects of SOD on EDR in aortae of WKY which had been partially inhibited by L-NOARG. As shown in Fig. 3, the ACh-induced EDR of WKY produced in the presence of 3×10^{-6} M L-NOARG was identical to the EDR produced in SHRSP in the absence of L-NOARG. In the presence of 3×10^{-6} M L-NOARG, the amplitude of EDR produced in aortae from WKY was again increased by 300 units/ml SOD (Fig. 5). Although the amplitude of EDR produced by each concentration of ACh was smaller in L-NOARG-treated aortae from WKY than in those from SHRSP, the maximum amplitude of EDR produced by 10^{-5} M ACh was $58.0 \pm 7.2\%$ ($n=12$) in the absence of SOD and $75.7 \pm 4.1\%$ ($n=10$) in the presence of SOD.

Discussion

Factors or mechanisms involved in EDR vary between different types of blood vessels (Shepherd and Katusic, 1991; Hwa *et al.*, 1994; Zygmunt, *et al.*, 1995; Shimokawa *et al.*, 1996; Berman and Griffith, 1998; Tare *et al.*, 2000). The present results, that the ACh-induced EDR of

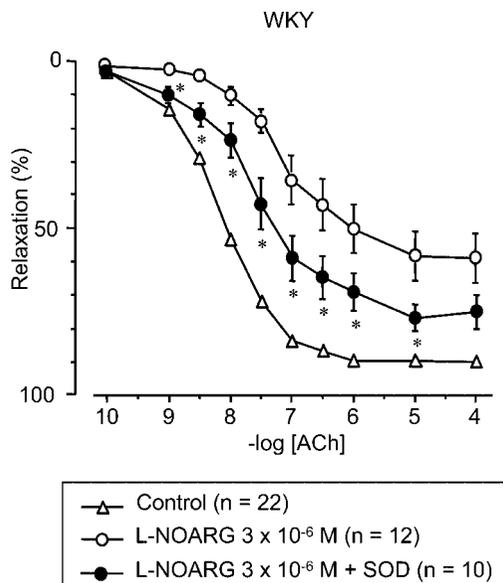


Fig. 5. Effects of SOD on the endothelium-dependent relaxation produced by ACh in aorta from WKY, in the presence L-NOARG (3×10^{-6} M). Aortic rings isolated from WKY were precontracted with noradrenaline (5×10^{-7} M), and ACh (10^{-10} – 10^{-4} M) was applied cumulatively, in the absence (open triangles) and presence of 3×10^{-6} M L-NOARG (open circles) and additional presence of both L-NOARG and 300 units/ml SOD (filled circles). Amplitude of relaxation relative to that of noradrenaline contraction is shown (mean \pm S.D.). Statistical significance of values obtained in the presence of both L-NOARG and SOD to those in the presence of L-NOARG alone was tested (*: $P < 0.05$).

both WKY and SHRSP aortae was blocked completely by removal of the endothelium or by application of 10^{-4} M L-NOARG, indicate that the EDR is induced by endothelial NO (Sekiguchi *et al.*, 1996). It has been reported that NO is inactivated by superoxide, and thus superoxide may impair EDR of vascular smooth muscle (Gryglewski *et al.*, 1986; Rubanyi and Vanhoutte, 1986; Laight *et al.*, 1998). In the present experiments, the EDR of NA-contracted aortae was shown to be impaired in preparations from SHRSP, confirming a number of previously reported observations (Lüscher, 1988, and also a review by Sunano *et al.*, 2000). Several mechanisms have been considered to be involved in the impairment of EDR in SHRSP, such as 1) reduced production of NO, 2) increased release of endothelium-derived relaxing factors such as endothelium-derived contractile factor (EDCF) and endothelium-derived hyperpolarizing factor (EDHF) or 3) diminished endothelium-dependent hyperpolarization of the smooth muscle membrane. Among these, the reduced release of NO may be the major mechanism for the reduction of EDR in aortae of SHRSP, since the EDR was mainly produced by the L-NOARG sensitive component. This agrees with previous reports that the release of NO is reduced in the aortic endothelium of SHRSP (Malinski *et al.*, 1993; Hirata *et al.*, 1996).

A reduced NO-component of EDR in SHRSP may be produced either by a decreased release of NO from the endothelium or by the increased destruction of NO, since no significant

difference in the relaxation of aortic smooth muscle to NO donors is observed between WKY and SHRSP (Sunano *et al.*, 2000). Increased destruction of NO may be produced by an interaction between NO and superoxide (Gryglewski *et al.*, 1986; Palmer *et al.*, 1987; Laight *et al.*, 1998). In fact, Grunfeld *et al.* (1995) observed that the release of NO decreased in the endothelial cells from SHRSP and the treatment of rats with SOD increased the release of NO. Pathogenesis of superoxide anion in the initiation of hypertension has also been reported in conventional SHR (Nakazono *et al.*, 1991). Thus, it is reasonable to consider that the increased release of superoxide anion may be one of the responsible mechanisms for the reduced release of NO in SHRSP. Scavenging superoxide by SOD may improve the impaired EDR by the destruction of NO in hypertensive rats. The present experiments confirmed that these were indeed the case in the aorta of SHRSP.

The present experiments showed that in WKY the reduced EDR with L-NOARG was partially recovered by treatment with SOD. The improved EDR by treatment with SOD has been reported in the rat aorta treated with a SOD inhibitor, diethyldithiocarbamate (Laight *et al.*, 1998) and also in the L-NOARG-treated cerebral artery (Rosenblum *et al.*, 1992). These results suggest that the effects of SOD would be much more pronounced when production of NO is impaired. The present results showed that SOD showed no effect on EDR in aortae from WKY, and these agree with the report that the enhancing effect of SOD on the release of NO was less in the endothelial cells of WKY, although the release of NO even in the absence of SOD was higher than that of SHRSP in the presence of SOD (Grunfeld *et al.*, 1995). Thus, the present results confirm the functional alteration of aortic tissues in SHRSP, as was observed in the measurement of NO in endothelial cells of hypertensive animals.

The observation that the reduced EDR by L-NOARG was also restored by SOD could be interpreted by assuming the involvement of superoxide in the actions of L-NOARG. Inhibition by SOD of the actions of L-NOARG to EDR has been reported in cerebral arterioles (Rosenblum *et al.*, 1992). Since the enhancement by SOD of EDR in WKY was not observed in the absence of L-NOARG, it is unlikely that superoxide is basically inactivating NO in this preparation. In the partial inhibition by L-NOARG of the production of NO, scavenging superoxide by SOD may augment EDR. These results indicate a possible augmentation of the production of superoxide by L-NOARG or the drug itself may increase the synthesis of superoxide. It is reported that NO synthase (NOS) activates the generation of superoxide and that the actions are blocked by N^o-nitro-substituted L-arginine (Heinzel *et al.*, 1992; Pou *et al.*, 1992). Taking these reports into consideration, such a possibility is unlikely. It is also reported that superoxide directly enhances the generation of NO (Hobbs *et al.*, 1994). An alternative explanation is that superoxide is synthesized in the aorta of WKY or as a result of stimulation by ACh, but that the amount of production is too little to alter the relaxation induced by endothelium-derived NO under control conditions, *i.e.*, in the absence of L-NOARG. In the partial inhibition by L-NOARG of the production of NO, the amount of superoxide may be sufficient to impair the relaxation.

It is suggested that the impairment of EDR in the aorta of SHRSP may be partly due to the reduction of effective NO due to its destruction by superoxide. Partial inhibition by L-NOARG of EDR produced in aortae from WKY may be caused, in part, by a mechanism similar to that found in SHRSP, *i.e.*, the reduction of NO due to its destruction by superoxide.

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(Received February 19, 2004; Accepted March 19, 2004)