Elimination of $^{\bullet}O_2^-/H_2O_2$ by α-lipoic acid mediates the recovery of basal EDRF/NO availability and the reversal of superoxide dismutase-induced relaxation in diabetic rat aorta

G. Koçak and Ç. Karasu

Department of Pharmacology, Faculty of Pharmacy, Ankara University, Ankara, Turkey

Aim: The aims of this study were to ascertain the mechanism(s) of relaxant action of exogenous superoxide dismutase (SOD) in aortic rings obtained from 12-week, streptozotocin(STZ)-diabetic and age-matched control rats, and to examine the effects of α-lipoic acid (ALA) treatment (for 6 weeks, after 6 weeks of untreated diabetes) on SOD-induced relaxations.

Materials and Methods: Thoracic aorta rings were suspended to isolated tissue chamber, and the changes in isometric tension were recorded.

Results: SOD produced a greater relaxation in untreated-diabetic rings compared with control rings. ALA treatment partially reversed SOD-induced relaxation in diabetic aorta. Pretreatment of rings with N^G-nitro-L-arginine methyl ester (L-NAME, 100 μM) inhibited SOD-induced relaxation. This effect of L-NAME was markedly observed in control and ALA-treated-diabetic rings compared with untreated-diabetic rings. SOD-induced relaxation was also inhibited by catalase (60 U/ml) in untreated-diabetic rings but not in ALA-treated-diabetic and control rings. Pretreatment with the cyclooxygenase inhibitor, indomethacin, or the catalase inhibitor, aminotriazole, had no effect on SOD-induced relaxation in any ring.

Conclusion: Findings suggested that: (i) in normal physiological conditions, the relaxant effect of SOD is related to the inhibition of superoxide anion radicals ($^{\bullet}O_2^-$)-induced endothelium-derived relaxing factor/nitric oxide (EDRF/NO) destruction in the rat aorta; (ii) in diabetic state, excess $^{\bullet}O_2^-$ increasingly inhibits basal EDRF/NO, and the dismutation of excess $^{\bullet}O_2^-$ to H_2O_2 is enhanced by exogenous SOD. H_2O_2 a vasorelaxant molecule, which probably accounts for the increased responsiveness of diabetic rings to exogenous SOD; and (iii) the reversal effect of *in vivo* ALA treatment on SOD-induced relaxation in diabetic aorta is probably linked with the elimination of $^{\bullet}O_2^-/H_2O_2$, which mediates the recovery of basal EDRF/NO availability.

Keywords: superoxide dismutase, rat aorta, streptozotocin-diabetes, nitric oxide, α-lipoic acid, hydrogen peroxide, endothelium

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Introduction

It is well known that the superoxide anion is an initial oxygen radical species, which may lead to secondary radicals or reactive oxygen species such as hydrogen peroxide (H_2O_2) or hydroxyl radical. Previous work from our laboratory suggested that the production of superoxide anion radicals ($^{\bullet}O_2^-$) and H_2O_2 is increased in diabetic vessels, and excess $^{\bullet}O_2^-$ may play a significant role in the inhibition of endothelium-derived relaxing factor/nitric oxide (EDRF/NO) bioavailability in basal physiological conditions [1–4]. We observed that the magnitude of endothelium-dependent relaxant response elicited by exogenous stimulants of NO synthase is variable, i.e. increased [1,5], unchanged [4] or decreased [2], depending on model and duration of

Correspondence:
Dr Çimen Karasu, Ankara University, Faculty of Pharmacy, Department of Pharmacology, Tandoğan 06100, Ankara, Turkey.
E-mail:
karasu@pharmacy.ankara.edu.tr

the experimental diabetes [1,2,4,5]. The increased vasorelaxant response to exogenous NO synthase stimulants might be a compensatory way (upregulation) in ambience in which basal activity of endothelial relaxation is decreased. We also suggested that raised $^{\bullet}O_2^-$ is responsible for increased transient nature of endothelium-dependent relaxation in diabetic vessels, and enhanced contribution of H_2O_2 in the establishment of stimulated endothelium-dependent relaxation might account for the maintenance of the NO synthase-mediated relaxation in diabetic arteries [6]. In this respect, the determination of vessel response to antioxidant enzymes such as superoxide dismutase (SOD) could be beneficial for the evaluation of functional abnormalities observed in diabetic vasculature. SOD catalyses the dismutation of $^{\bullet}O_2^-$ to H_2O_2. A previous study demonstrated that the addition of exogenous SOD to the pre-contracted rat aorta was able to produce relaxation, and this relaxation was significantly greater in diabetic than in control aorta [7]. In this study, therefore, we questioned *in vitro* the mechanism(s) of vascular action of exogenous SOD in diabetic and age-matched control rats.

On the other hand, the functional effects of antioxidant treatment include protection of endothelial function and vascular reactivity, and amelioration of blood pressure and vascular morphology [3,8–11]. α-Lipoic acid (ALA) is a naturally occurring free-radical scavenger and a transition metal chelator that activates glucose uptake [12–14]. α-Lipoic acid is also a co-factor for mitochondrial pyruvate dehydrogenase, and has been termed a 'metabolic antioxidant' [15]. In this study, we also investigated the effects of *in vivo* ALA treatment to understand better the underlying mechanisms of the vasorelaxant action of exogenous SOD, and the relationships among the spontaneously produced EDRF/NO, reactive oxygen species and the antioxidant ALA in diabetic animals.

Materials and Methods

Animals

Male Wistar rats were used in the experiments according to the proposals of the Declaration of Helsinki and the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. Diabetes was induced by a single injection of streptozotocin [STZ, 55 mg/kg, intraperitoneal (i.p.)], and confirmed from tail-vein blood glucose 48 h after STZ injection. Rats with blood glucose concentrations of 300 mg/dL or more were considered diabetic. The experimental groups included control and diabetic rats treated with or without ALA (50 mg/kg/day racemate, i.p, for 6 weeks, after 6 weeks of untreated diabetes). All rats were maintained under standard housing conditions for 12 weeks before the experiments were conducted. Blood glucose concentrations were measured by an Ames glucometer (Glucometer III, Bayer Diagnostics, France).

Isolation of Aortic Rings and Vascular Reactivity Studies

The descending thoracic aorta was quickly isolated, cleaned and sectioned into 3- to 4-mm-long rings as described previously [3,4,6]. The rings were suspended between parallel hooks in 5-ml tissue baths filled with Krebs–Henseleit bicarbonate buffer solution, which were thermoregulated at 37 °C. Changes in isometric tension were recorded on an Ugo Basile recorder via Ugo Basile-7004 transducer (Varese, Italy). The rings were equilibrated for 60 min under a resting tension of 2 g before experiments were begun. After equilibration, concentration–response curves to increasing concentrations of phenylephrine were performed on each ring. The rings were then contracted with a submaximal concentration of phenylephrine (EC_{80}, this was ≈1 μM). At the peak of contraction, a cumulative concentration–response curve for SOD was obtained. Some rings were pre-incubated with L-NAME (100 μM, 20 min), indomethacin (1 μM, 20 min) catalase (60 U/ml, 30 min) or aminotriazole (5 mM, 30 min), and then the relaxation to a single concentration of SOD (150 U/ml) was determined.

Chemicals and Data Analysis

All chemicals used in experiments, except α-lipoic acid, were purchased from Sigma Chemical (St. Louis, MO, USA). α-Lipoic acid was obtained from Fluka BioChemika (Switzerland).

Relaxation to SOD was expressed as a percentage decrease of the phenylephrine-induced contraction. Data are presented as group means ± s.e.m. They were given Bartlett's test for homogeneity of variances, followed by log transformation when appropriate, before being subjected to one-way analysis of variance. When overall significance ($p < 0.05$) was attained, between groups differences were established by *post hoc* analysis using the Bonferroni test, which is corrected for multiple comparisons.

Results

General Characteristics of Animals

Diabetic rats exhibited loss of body weight, and ALA treatment led to an increase in body weights of diabetic animals (table 1). However, at the end of the treatment

Table 1 The body weights and blood glucose levels of animals (at the beginning of the study: two days after STZ injection)

	Control (n = 10)	Untreated-diabetic (n = 10)	ALA-treated-diabetic (n = 12)
Body weight (g)			
At the beginning of the study	284 ± 5	276 ± 9	280 ± 7
At the beginning of the ALA treatment	322 ± 14	228 ± 9***	230 ± 7***#
At the end of the ALA treatment	336 ± 10	204 ± 13***	261 ± 13***†
Blood glucose (mmol/l)			
At the beginning of the study	5.4 ± 0.3	22.3 ± 0.8***	20.4 ± 0.9***#
At the beginning of the ALA treatment	5.6 ± 0.2	23.5 ± 0.9***	22.8 ± 0.7***###
At the end of the ALA treatment	5.7 ± 0.3	24.7 ± 1.1***	16.7 ± 1.1***†††

***$p < 0.001$ vs. control; †$p < 0.05$, †††$p < 0.001$ vs. untreated-diabetic; #$p < 0.05$, ###$p < 0.001$ vs. at the end of the ALA treatment.

period, the body weights of untreated-diabetic rats were still significantly lower than those of the control rats. All diabetic rats showed hyperglycaemia (table 1). The 6-week ALA treatment induced a significant fall in blood glucose concentrations of diabetic animals. However, blood glucose concentrations of ALA-treated-diabetic rats were found to be significantly higher than in control rats (table 1).

SOD-induced Relaxations and the Effects of Indomethacin, L-NAME, Catalase or Aminotriazole

In rings with intact endothelium, SOD produced concentration-dependent relaxation that was augmented significantly in diabetic rings compared with control and ALA-treated rings (figure 1a). As demonstrated previously [7], the inhibition of cyclooxygenase activity by indomethacin had no effect on SOD-induced relaxation in either control or diabetic rings (not shown). Although pretreatment with L-NAME significantly inhibited SOD-induced relaxation in all experimental groups (figure 1b), the percentage inhibition ratio calculated from the magnitude of SOD-induced relaxation before and after L-NAME incubation in untreated-diabetic rings (65.4 ± 1.7) was significantly less ($p < 0.01$) than that obtained from control (87.8 ± 3.1) and ALA-treated-diabetic rings (89.5 ± 1.1). Pretreatment with catalase inhibited SOD-induced relaxation in control rings slightly but not significantly (figure 1b). The percentage inhibition ratio of SOD-induced relaxation obtained after catalase incubation in untreated-diabetic aorta was 62.2 ± 3.8, which was significantly higher than the corresponding values taken from the control (18.1 ± 1.5; $p < 0.001$) and ALA-treated-diabetic rings (13.2 ± 2.2; $p < 0.001$) (figure 1b). SOD-induced relaxation did not change by pretreatment with aminotriazole either in control, untreated or ALA-treated-diabetic rings (figure 1b). As shown in figure 1a and b, the increased responsiveness of diabetic aorta to exogenous SOD was improved significantly by 6-week ALA treatment ($p < 0.05$ vs. control; $p < 0.05$ vs. untreated-diabetic).

Discussion

This study confirmed the results of a previous investigation, which showed that the addition of exogenous SOD to pre-contracted rat aorta produced concentration-dependent relaxation that was increased in STZ-diabetic rats [7]. Because the relaxant effect of SOD in rings from non-diabetic control animals was markedly inhibited by pretreatment with a NO synthase inhibitor (but not with catalase) and $^{\bullet}O_2^-$ is known to inactivate EDRF/NO, we concluded that SOD-induced relaxation is closely related to the inhibition of $^{\bullet}O_2^-$-dependent destruction of spontaneously produced EDRF/NO in normal physiological conditions. This interpretation is parallel with that of Langenstroer and Pieper [7]. They suggested that the removal of inhibitory action of overproduced $^{\bullet}O_2^-$ on increased spontaneously liberation of EDRF/NO is the possible mechanism for increased vasorelaxant action of exogenous SOD in diabetic vessels, and that the basal production of EDRF/NO is increased in diabetic rats [7]. But, our findings do not totally support their suggestion, as we found that the mean percentage inhibition ratio calculated from the SOD relaxations determined separately in rings untreated or pretreated with L-NAME was significantly less in untreated-diabetic animals compared with control or ALA-treated-diabetic animals. This finding implies that: (i) diabetes leads to inhibition of the basally production and/or activity of EDRF/NO; and (ii) in addition to EDRF/NO, a substance or factor is also involved in the generation of vasorelaxant response to exogenous SOD in diabetic vessels. It is improbable that this will have a prostanoid factor because pretreatment with indomethacin did not change SOD-induced relaxation in either control or

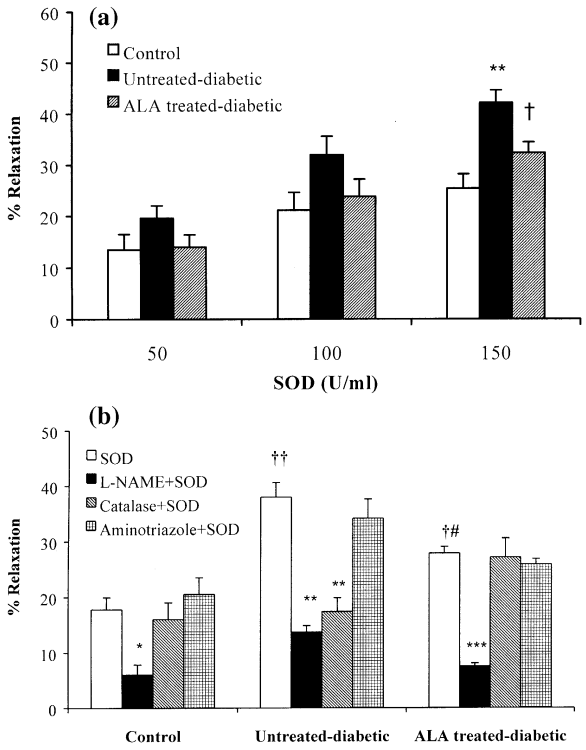

Fig. 1 (a) This figure shows the relaxations to cumulatively increasing concentration of superoxide dismutase (SOD) in precontracted aortic rings obtained from non-diabetic control ($n = 10$), untreated-diabetic ($n = 10$) and α-lipoic acid (ALA)-treated-diabetic ($n = 12$) rats. $^{**}p < 0.01$ vs. control. $\dagger p < 0.05$ vs. untreated-diabetic. (b) This shows the effect of pretreatment with L-NAME (100 μM), catalase (60 U/ml) or aminotriazole (5 mM) on SOD (150 U/ml)-induced relaxations in every experimental group. The bars express a single dose of SOD-(150 U/ml)-induced relaxation in untreated rings (SOD; $n = 10$ for non-diabetic control; $n = 10$ for untreated-diabetic and $n = 12$ for α-lipoic acid (ALA)-treated-diabetic rats), in rings pretreated with L-NAME (L-NAME + SOD; $n = 8$ for non-diabetic control; $n = 7$ for untreated-diabetic and $n = 7$ for ALA-treated-diabetic rats), in rings pretreated with catalase (catalase + SOD; $n = 7$ for non-diabetic control; $n = 7$ for untreated-diabetic and $n = 8$ for ALA-treated-diabetic rats) or in rings pretreated with aminotriazole (aminotriazole + SOD; $n = 8$ for non-diabetic control, $n = 8$ for untreated-diabetic and $n = 8$ for ALA-treated-diabetic rats). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. SOD within group. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ vs. non-diabetic control rats. $\#p < 0.05$ vs. untreated-diabetic rats.

diabetic rats. However, SOD-induced relaxation in aorta was significantly inhibited by catalase incubation in untreated-diabetic animals but not in control and ALA-treated animals. This finding suggests the involvement of H_2O_2 in SOD-induced relaxation in diabetic vessels. Indeed, the production of H_2O_2 may increase by the addition of exogenous SOD to the bath medium, when endogen production of $^{\bullet}O_2^-$ was already increased. As indicated previously by others [16] and ourselves [4,6], this study confirms the increased vascular production of $^{\bullet}O_2^-$ in diabetic vessels. In accordance with the present findings, we showed previously that H_2O_2 produces relaxation in pre-contracted rat aorta by either direct stimulation of guanylate cyclase or the stimulation of EDRF/NO release [6]. We also showed that the vasorelaxant action and the spontaneous production of H_2O_2 were significantly increased in diabetic rats [6].

As a novel finding, this study demonstrated that *in vivo* ALA treatment is able to improve SOD-induced relaxation in diabetic rat aorta. If we accept the facts that (i) the increased spontaneous production of $^{\bullet}O_2^-$ results in enhanced destruction of basal EDRF/NO in diabetic vessels; and (ii) the exogenous SOD preserves basal EDRF/NO against the destructive effect of $^{\bullet}O_2^-$ and also leads to an increase in vessel H_2O_2 content in diabetic rats, thus our finding suggests that ALA treatment inhibits the over production of $^{\bullet}O_2^-$ and improves diabetes-induced deficiency in basal EDRF/NO activity, as do several antioxidants including vitamin E and probucol [3,8,9]. Indeed, L-NAME-induced inhibition in SOD-induced relaxation was observed to the similar extent in control and ALA-treated-diabetic animals, indicating the recovery of basal EDRF/NO availability. This is consistent with the results of our recent studies, showing that *in vivo* ALA treatment results in morphological improvement in aortic endothelial cells of diabetic rats [10,11]. In accordance with this, the diabetes-induced endothelial deficiency and the impaired NO-mediated relaxation of corpus cavernosum smooth muscle have been demonstrated to ameliorate or reverse by *in vivo* ALA treatment [17,18]. ALA treatment has also been shown to protect cultured endothelial cells against the advanced glycosylation end-products-induced oxidative stress [19]. Many *in vitro* or *in vivo* studies have shown that ALA scavenges hydroxyl radicals, hyperchlorous acid, H_2O_2 and singlet oxygen, and repairs oxidative damage [20]. Tissues can convert ALA to dihydro-ALA, which has a broader spectrum of action than ALA, with additional scavenging $^{\bullet}O_2^-$ and peroxyl radicals [20]. In this regard, the eliminating the excess $^{\bullet}O_2^-$ by *in vivo* ALA treatment seems to be essential for the amelioration of EDRF/NO bioavailability in diabetic vessels. On the other hand, the ALA-dyhdro-ALA cycle may recycle major antioxidants including glutathione, ascorbate, thioredoxin and ubiquinone. ALA also chelates transition metals, thus reducing free radical formation by glucose autoxidation [13]. Previous studies have indicated a beneficial role of chronic treatment with

free-radical scavengers or metal chelators in the prevention of diabetes-induced endothelial dysfunction [21,22]. This study demonstrated that catalase incubation was unable to block SOD-induced relaxation in rings from ALA-treated-diabetic animals, indicating removal of contribution of H_2O_2 in exogenous SOD-induced relaxation when the rats were treated with ALA. This is consistent with the previous studies showing that *in vivo* ALA treatment inhibits intracellular H_2O_2 production in erythrocytes [11], and improves catalase activity of diabetic vessels [10,11].

Some previous studies have demonstrated that acute or chronic ALA treatment influences vascular endothelial and neural functions without an effect on glycaemic control or body weight [17,18,23]. However, our present or recent studies showed that the improving effects of *in vivo* ALA treatment on increased blood pressure and vascular reactivity, and impaired EDRF/NO availability accompany its ameliorating effects on oxidative stress, glucose and lipid metabolism in diabetic rats [10,11]. The discrepancy between the results of our studies and others is not known, but may be related to the dose of ALA or the manner and duration of the treatment [17,18]. However, the normalization of uptake, transport and utilization of glucose by *in vivo* ALA treatment has been reported previously in diabetic animals [14]. ALA is able to enhance non-oxidative and oxidative glucose metabolism, and induces hypoglycaemia [24]. In fact, the mechanism of action of ALA on vascular–endothelial recovery might be more intricate than recognized, as previous studies have reported that ALA improves energy metabolism, mitochondrial and cytoplasmic NAD^+:NADH ratios, normalizes (Na^+, K^+)-ATPase activity and the tissue contents of reduced glutathione, myo-inositol and taurin, and protects cytocrom b_5 reductase in various tissues of diabetic patients or animals [23,25–27]. Taurin has been reported to restore diabetes-induced endothelial dysfunction and vasoconstriction [28]. Furthermore, a 'NADPH-sparing' effect of ALA could also benefit endothelial function because NADPH is a co-factor for NO synthase [17].

In summary, the present study provided the evidence that exogenous SOD-induced relaxation depends on the inhibition of $^{\bullet}O_2^-$-induced EDRF/NO destruction in normal physiological conditions, but, in a diabetic state, raised $^{\bullet}O_2^-$ decreases basal EDRF/NO, and exogenous SOD catalyses the dismutation of raised $^{\bullet}O_2^-$ to H_2O_2 that probably accounts for an increased relaxant response to SOD. This study also demonstrated that a metabolic antioxidant, ALA, is able to improve diabetic deficit in basal EDRF/NO availability. Although the present findings imply the $^{\bullet}O_2^-/H_2O_2$ elimination in ALA-mediated EDRF/NO improvement, we believe that ALA effects on vascular recovery are probably more complex than anticipated for a simple free-radical scavenger in line with the observations.

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