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Changes in superoxide dismutase mRNA expression by streptozotocin-induced diabetes

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1 Experiments were designed to investigate the involvement of superoxide anions in the attenuated endothelium-dependent relaxation of the rat aorta from streptozotocin (STZ)-induced diabetic rats.

2 The endothelium-dependent relaxation responses to acetylcholine (ACh, 10^{-7} M) in helical strips of the aorta precontracted with noradrenaline (NA, $5 \times 10^{-8} \sim 3 \times 10^{-7}$ M) were significantly decreased in STZ-induced diabetic rats. The recovery phase of the relaxation after single administration of ACh in the STZ-induced diabetic rats was more rapid than those in control vessels.

3 Preincubation of aortic strips with superoxide dismutase (SOD, 60 u ml^{-1}) normalized the recovery phase of the relaxation of diabetic aorta after single administration of ACh, whereas catalase (150 u ml^{-1}) or indomethacin (10^{-5} M) had no effects on the relaxation.

4 SOD (180 u ml^{-1}) caused relaxation in NA precontracted aortic strips and the degree of the SOD-induced relaxation was significantly greater in diabetic aorta as compared with age-matched control vessels.

5 When the changes in mRNA expressions of Mn-SOD or Cu-Zn-SOD were observed, Mn-SOD mRNA expression was markedly decreased, and Cu-Zn-SOD was slightly decreased in diabetic aorta.

6 These results suggest that the rapid destruction of NO by superoxide anions may occur in the STZ-induced diabetic rats, and this may be due to a decrease in mRNA expression of Mn-SOD or Cu-Zn-SOD.

Keywords: Diabetes; endothelium; O_2^-; superoxide dismutase; mRNA

Introduction

Vascular disease is one of the complicating features of diabetes-mellitus in man (Christrieb, 1973). An accumulating body of evidence indicates that the relaxation responses of aortic strips to endothelium-dependent agents are decreased in streptozotocin (STZ)-induced diabetic rats (Oyama *et al.*, 1986; Pieper & Gross, 1988; Kamata *et al.*, 1989a,b; Tomlinson *et al.*, 1992; Abiru *et al.*, 1993; Poston & Taylor, 1995).

Nitric oxide (NO) has been proposed as the major form of the endothelium-derived relaxing factor and contributes to arterial vasodilatation (Moncada *et al.*, 1991). Recently, the interaction between NO and superoxide anions has received a great deal of attention. Since NO is rapidly inactivated by superoxide anions, it has been suggested that an enhanced formation of this radical species may be involved in the accelerated breakdown of NO (Gryglewski *et al.*, 1986; Rubanyi & Vanhoutte, 1986a; Mian & Martip, 1995). Indeed, Hattori *et al.* (1991) showed that an enchanced fade of endothelium-dependent relaxation in diabetes may stem from a greater production of superoxide anions presumably due to reduced superoxide dismutase activity. The importance of increased free-radical synthesis in abnormal endothelial function in diabetes is strengthened by the observation that a variety of pharmacological free-radical scavengers, including superoxide dismutase (SOD), desferrioxamine and allopurinol, improve the endothelial function in arteries from diabetic animals (Hattori *et al.*, 1991; Tesfamariam & Cohen, 1992; Langenstroer & Pieper, 1992; Pieper *et al.*, 1992) and in normal arteries incubated in a medium containing an elevated concentration of glucose (Tesfamariam & Cohen, 1992; Taylor & Poston, 1994), In addition, Pieper *et al.* (1992) have demonstrated, using a bioassay technique for endothelium-derived relaxing factor, that free radicals mediate the destruction of NO in diabetic rat aorta.

There are few data which have directly assessed the relationship between the mRNA expression in SOD and the destruction of NO in endothelial dysfunction in diabetes. To our knowledge, no studies have investigated mRNA expression in SOD in animal models of diabetes. The competitive reverse-transcription polymerase chain reaction (RT-PCR) could be used as a reliable method to quantify RNA levels since the sensitivity and the accuracy of this quantitative PCR method has been demonstrated previously (Porcher *et al.*, 1992; Pannetier *et al.*, 1993). An internal standard (competitor RNA) of known concentration was reverse transcribed with the target RNA and then co-amplified by using identical primers in the same tube. In this reaction, the parameters controlling the reverse-transcription and the amplification efficiencies should affect similarly both templates (Wang *et al.*, 1989; Gilliland *et al.*, 1990; Santiard-Baron *et al.*, 1995).

The purpose of the present study was to define the relationship between changes in mRNA expression in SOD and endothelial dysfunction in STZ-induced diabetic rats.

Methods

Animals and experimental design

Male Wistar rats, 8 weeks old and 220-250 g in weight, received a single injection in the tail vein of STZ 60 mg kg^{-1}, dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given *ad libitum*. The concentration of glucose in plasma was determined by the *O*-toluidine method (Dubowski, 1962). This study was conducted in accordance with the Guide for the Care and Use of Laboratory Animals adopted by the Committee on the Care and Use of Laboratory Animals of Hoshi University which is accredited by the Ministry of Education, Science, Sports and Culture, Japan.

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Measurement of isometric force

Ten weeks after treatment with STZ or buffer, rats were killed by decapitation. A section of the thoracic aorta between the aortic arch and the diaphragm was then removed and placed in oxygenated, modified Krebs-Henseleit solution (KHS). The solution consisted of (mM): NaCl 118.0, KCl 4.7, $NaHCO_3$ 25.0, $CaCl_2$ 1.8, NaH_2PO_4 1.2, $MgSO_4$ 1.2, dextrose 11.0. The aorta was cleaned of loosely adhering fat and connective tissue and cut into helical strips 3 mm in width and 20 mm in length. The tissue was placed in a well-oxygenated (95% O_2, 5% CO_2) bath of 10 ml KHS at 37°C with one end connected to a tissue holder and other to a force-displacement transducer (Nihon Kohden, TB-611T). The tissue was equilibrated for 60 min under a resting tension of 1.0 g (preliminary determined to be optimum). During this time the KHS in the tissue bath was replaced every 20 min.

After equilibration, each aortic strip was contracted with 10^{-7} M noradrenaline (NA). The presence of functional endothelial cells was confirmed by demonstrating relaxations to 10^{-5} M acetylcholine (ACh), and aortic strips in which 75% relaxation occurred were regarded as tissues with endothelium. The relaxation responses to ACh and SOD were expressed as a percentage of the contractile force induced by 10^{-7} M NA. Since the maximal contraction of aortic strips in response to NA was enhanced in diabetic rats (Kamata *et al.*, 1988), for the relaxation studies, the aortic strips were precontracted with an equieffective concentration of NA ($5\times10^{-8}\sim3\times10^{-7}$ M). This concentration produced 75–85% of the maximal response. Aortic strips, which were weighed at the end of each experiment, were precontracted with $5\times10^{-8}\sim3\times10^{-7}$ M NA so that the strips developed a tension of approximately 95 mg mg^{-1} tissue in both age-matched control and diabetic rats. When the NA-induced contraction reached a plateau level, the relaxant response to ACh (10^{-7} M) or SOD (180 u ml^{-1}) was examined in a single concentration-effect manner. Each aortic strip was exposed to only one concentration of an agonist.

When the effects of various scavengers of free radicals or indomethacin on the response to the relaxant agents were examined in age-matched control and diabetic aorta, each scavenger or indomethacin was added to the bath 20 min before the administration of NA.

Measurement of mRNA expression in SOD

Oligonucleotides The following oligonucleotides (ON) were used as primers for RT–PCR with the respective EMBL data library accession number and position of the PCR product in the coding sequence in brackets: rat GAPDH (X02231, position 492-799) ON 1; 5′-TCCCTCAAGATTGTCAGCAA-3′, ON 2; 5′-AGATCCACAACGGATACA TT-3′: rat Cu-Zn-SOD (X05634, position 58-505) ON 3; 5′-GCAGAAGGCAAGCGGTGAAC-3′, ON 4; 5′-TAGCAGGACAGCAGATGAGT-3′; rat Mn-SOD (Y00497, position 329-945) ON 5: 5′CCCTAAGGGTGGTGGAGAAC-3′, ON 6; 5′-GGCCTTATGATGACAGTGAC-3′.

RNA isolation and RT–PCR

RNA was isolated according to the guanidinium method (Chomczynski & Sacchi, 1987). Rat aortae were carefully isolated and cleaned of adhering parenchyma and connective tissue. The tissue was homogenized in RNA buffer using a glass homogenizer. For the RT–PCR analysis, first-strand cDNA was synthesized from total RNA by use of Oligo $(dT)_{12\text{-}18}$ and cDNA SYNTHESIS KIT (LIFE SCIENCES, INC). RNA (1 μg) was reversely transcribed in a final volume of 20 μl with 12.5 unit AMV-reverse transcriptase in first-strand reaction mix, 12.5 mM dithiothreitol (DTT), 0.05 μg oligo $(dT)_{12\text{-}18}$, and 12.5 units RNasin RNase inhibitor for 1 h at 42°C, for 7 min at 99°C. Twenty five PCR cycles (55°C for 1 min, 72°C for 1 min, 94°C for 1 min) were performed in a final volume of 50 μl with half of the reverse transcription (RT) mixture, 0.4 μM of each primer, 0.4 μM of each GAPDH primer as an internal control, 0.4 mM dNTP (BRL), and 2.5 units Taq-DNA-polymerase (BRL). The obtained PCR products were analyzed on ethidium bromide-stained agarose (1.5%) gels.

Competitive PCR

An amount of mRNA was measured with competitive PCR techniques by use of a heterogeneous DNA fragment (PCR mimic) as an internal standard. PCR mimic was created essentially according to the instructions of a kit (PCR MIMIC Construction kit, Clontech, CA, USA). A part of the heterogeneous DNA fragment was amplified with protruding 20 bp in ends specific for Cu-Zn-SOD or Mn SOD primers, respectively. In the second round of PCR, an aliquot of the first PCR product was amplified again with the target gene specific primers for Cu-Zn-SOD or Mn SOD. Two μl of serial dilution of the PCR mimic (10, 1, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} attomol μl^{-1}) was added to PCR amplification reaction (25 cycles) containing a constant amount of the rat cDNA sample. To determine the exact amount of the target mRNA species, two fold dilutions of the PCR mimic (10, 5, 2.5, 1.25, 0.625, 0.3125 attomol μl^{-1}) were used for the PCR amplification. Ten percent of the RT mixture with 4 μg of original RNA, obtained from control or diabetic rat, were amplified by PCR in 25 cycles with serial dilution of the PCR mimic. A semiquantitive evaluation of mRNA levels was analyzed by comparing each product (mimic cDNA Target DNA) after electrophoreses.

Drugs

Streptozotocin, (−)-noradrenaline hydrochloride, superoxide dismutase, catalase and indomethacin were purchased from Sigma Chemicals Co. (St. Louis, MO, U.S.A.). Acetylcholine chloride was purchased from Daiichi Pharmaceuticals (Tokyo, Japan). All drugs were dissolved in saline. All concentrations are expressed as final molar concentrations of the base in the organ bath.

Statistical analysis

Data are expressed as the mean ± s.e.mean. Statistical differences were measured by Student's *t* test for unpaired observations, following one-way analysis of variance and considered significant if $P<0.05$.

Results

Ten weeks after treatment with STZ, the concentration of glucose in plasma was elevated significantly, from 114.6 ± 3.4 (mg dl^{-1}) in age-matched controls to 523.9 ± 17.9 (mg dl^{-1}) in diabetic rats, respectively.

Effects of various agents on the time course of the relaxation induced by ACh

ACh (10^{-7} M) caused a rapid and long-lasting relaxation of age-matched control aortic strips and the maximal relaxation was 78.9 ± 2.8% ($n=6$) as shown in Figure 1a. The relaxant responses to ACh (10^{-7} M) were more transient in diabetic rats, and the maximal relaxation was significantly decreased (65.2 ± 4.0%, $n=6$, $P<0.05$). These data are summarized in Figure 1b.

Preincubation with SOD (60 u ml^{-1}), indomethacin (10^{-5} M) or catalase (150 u ml^{-1}) had no effect on 10^{-7} M ACh-induced relaxation of age-matched control aortic strips (Figure 2a). Preincubation with SOD (60 u ml^{-1}) significantly augmented ACh-induced relaxation of diabetic aorta; the maximal relaxation was enhanced and the rapid

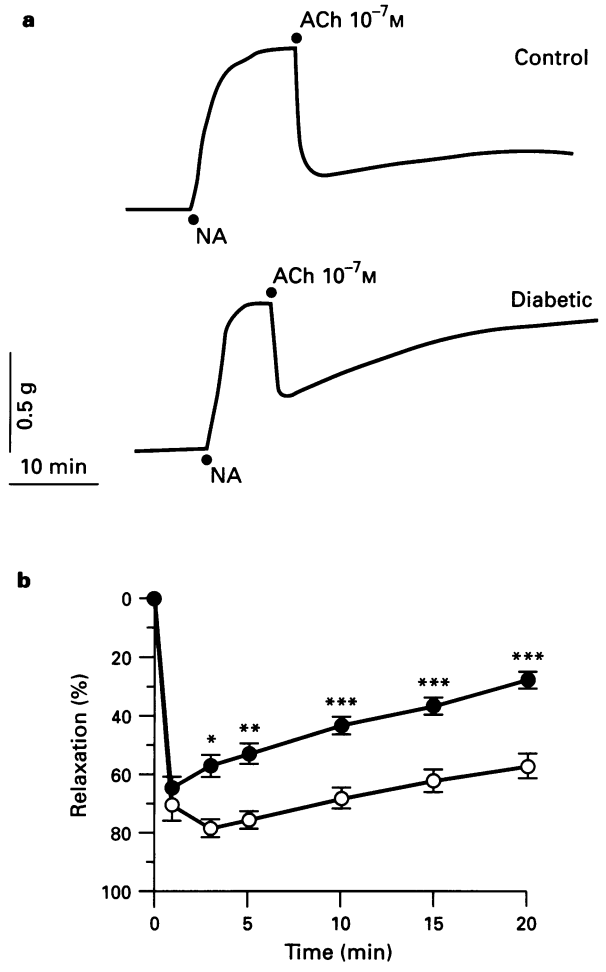

Figure 1 (a) Typical records showing the effects of acetylcholine (10^{-7} M)-induced relaxation in noradrenaline (NA, $5\times10^{-8}\sim3\times10^{-7}$ M)-precontracted aortic strips obtained from age-matched control and STZ-induced diabetic rats. (b) Time course of changes in relaxant responses to acetylcholine in aortic strips from age-matched control and STZ-induced diabetic rats. Age-matched control rats ($n=6$, ○); STZ-induced diabetic rats ($n=6$, ●). Values are mean±s.e. Significantly different from age-matched control, $*P<0.05$, $**P<0.01$, $***P<0.001$.

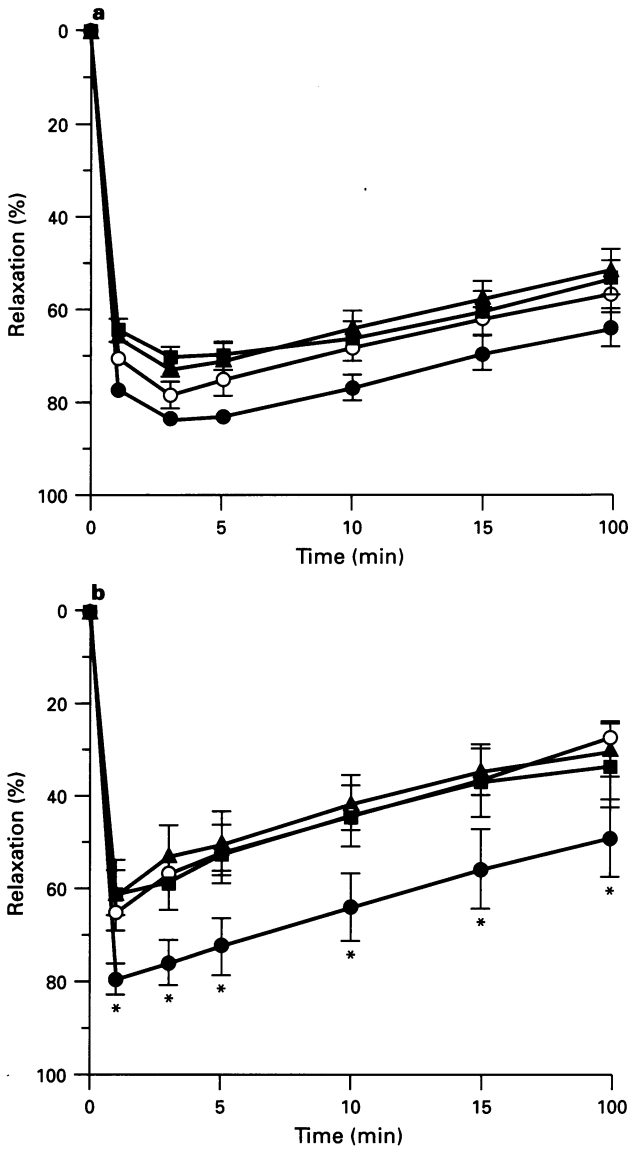

Figure 2 Effects of various agents on acetylcholine (10^{-7} M)-induced relaxation in noradrenaline ($5\times10^{-8}\sim3\times10^{-7}$ M)-precontracted aortic strips obtained from age-matched control (a) and STZ-induced diabetic (b) rats. Control ($n=6$, ○); treated with 60 u ml^{-1} SOD ($n=6$, ●); 10^{-5} M indomethacin ($n=6$, ▲); 150 u ml^{-1} catalase ($n=6$, ■). Values are mean±s.e. Significantly different from non-treated aortae, $*P<0.05$.

fade of the endothelium-dependent response observed in diabetic vessels was significantly improved by SOD as shown in Figure 2b. However, pretreatment with indomethacin (10^{-5} M) or catalase (150 u ml^{-1}) did not prevent the rapid fade of the endothelium-dependent relaxation. The combined application of indomethacin and catalase also had no effect on ACh-induced relaxation in STZ-induced diabetic vessels (data not shown). Neither SOD, indomethacin nor catalase affected the basal tonus of the aortic strips obtained from age-matched control and STZ-induced diabetic rats.

Effects of SOD on the aortic strips

High concentrations of SOD (180 u ml^{-1}) relaxed the NA-precontracted aorta in age-matched control rats and the maximal relaxation was 30.7±4.6% ($n=6$) as shown in Figure 3a. In STZ-induced diabetic aorta, the maximal relaxation of aortic strips by SOD (180 u ml^{-1}) was significantly increased (48.2±5.2%, $n=6$, $P<0.05$), and the relaxation response was of longer duration than in age-matched control aorta. These data are summarized in the lower panel of Figure 3b. SOD-induced relaxation was not changed by preincubation with catalase (150 u ml^{-1}) (data not shown) in the STZ-induced diabetic rats.

mRNA expression in Mn SOD or Cu-Zn-SOD

Using RT–PCR in the total RNA isolated from diabetic and age-matched control aorta, we examined the changes in mRNA expression in Mn-SOD or Cu-Zn-SOD. When the changes in mRNA expression in Mn-SOD or Cu-Zn-SOD existing in the cells were observed, Mn-SOD mRNA expression was markedly decreased, and Cu-Zn-SOD was slightly decreased in diabetic aorta (Figure 4). A semi-quantitative evaluation of mRNA levels was analyzed by comparing each product after electrophoresis. By this method, we found that Cu-Zn-SOD was slightly decreased and Mn-SOD mRNA was significantly decreased in the diabetic aorta (Figure 5).

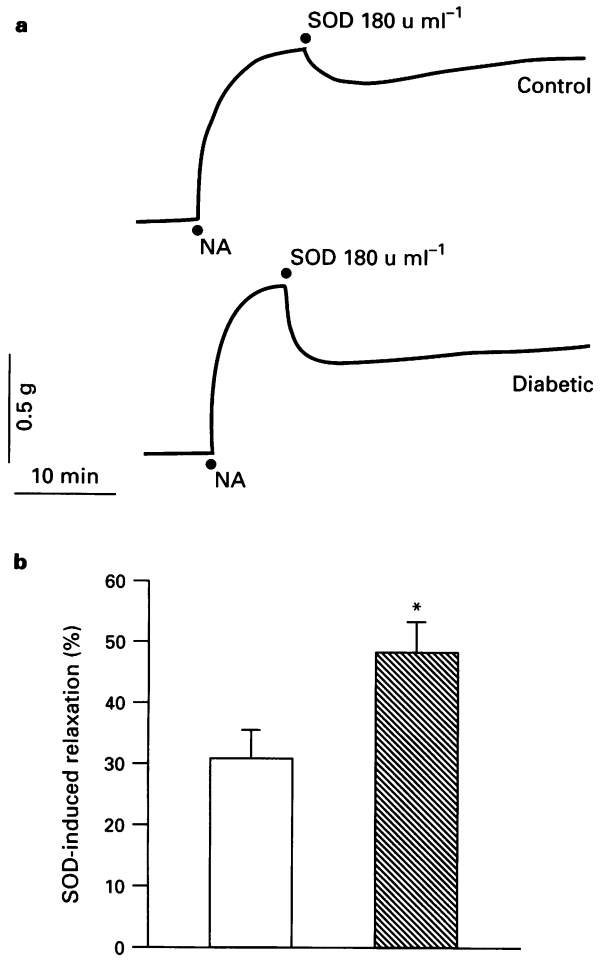

Figure 3 (a) Typical records showing the effects of SOD (180 u ml^{-1})-induced relaxation in noradrenaline (NA, $5 \times 10^{-8} \sim 3 \times 10^{-7}$ M)-precontracted aortic strips obtained from age-matched control and STZ-induced diabetic rats. (b) The data in (a) are summarized. Age-matched control rats ($n=6$, open column); STZ-induced diabetic rats ($n=6$, hatched column). Values are mean ± s.e.; $n=6$ animals. Significantly different from age-matched control, $^*P<0.05$.

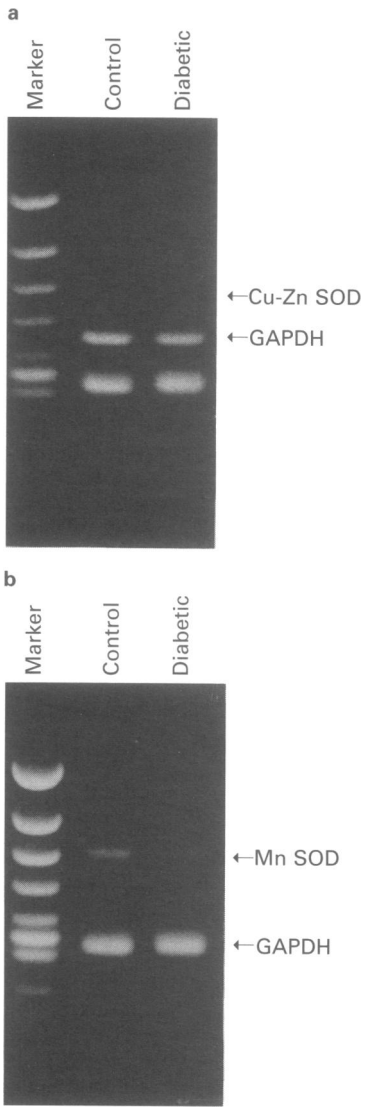

Figure 4 RT-PCR assay of SOD mRNA expression in control and diabetic rat aorta. (a) Cu-Zn-SOD mRNA expression (447 bp); (b) Mn-SOD mRNA expression (616 bp). Total RNA was extracted as described in Methods. Each total RNA preparation (1.0 μg) was reverse transcribed and half of the cDNA products was PCR-amplified with each primer, for 25 cycles. A portion of the PCR reaction product was electrophoresed on a 1.5% agarose gel containing ethidium bromide.

Discussion

The main conclusion from the present study is that rapid destruction of NO by superoxide anion occurs in the STZ-induced diabetic rats, and this may be due to the decreased content of SOD.

A prior incubation of aortic-strips with SOD normalized the maximal relaxation response and the recovery phase of the relaxation of diabetic aorta after a single administration of ACh, whereas catalase or indomethacin had no effect on the relaxation. The protective effect of SOD on the diabetes-induced impairment of ACh-induced maximal relaxations and the duration of relaxations indicates a role for oxygen-derived free radicals in the blunted response. In agreement with our findings, SOD has been shown to improve endothelium-dependent relaxation in diabetic rat aorta (Hattori *et al.*, 1991), glucose-induced impairment of ACh relaxation in rabbit aorta (Tesfamariam & Cohen, 1992) which occurs as a result of increased cyclo-oxygenase activity, and glucose-induced impairment of ACh relaxation in isolated mesenteric resistance artery (Taylor & Poston, 1994) which includes a role for increased free radical production, polyol pathway activation and altered L-arginine metabolism. Since catalase did not affect the enhanced transient nature of the endothelium-dependent relaxation in diabetic vessels in the present study, it is unlikely that hydrogen peroxide and hydroxyl radicals are involved. It has been shown, however, that other radicals may also be involved because catalase, a scavenger of hydrogen peroxide, and deferoxamine, which prevents the formation of hydroxyl radicals, also may restore abnormal diabetic endothelial cell function (Tesfamariam & Cohen, 1992). Other studies with STZ-induced diabetic rat aorta (Hattori *et al.*, 1991) have demonstrated that hydroxy radicals are not involved in endothelial dysfunction. These inconsistent results may be partly related to differences in experimental models used, i.e., differences in species, strains, vessel types, ages of animals, and duration of diabetes.

SOD was able to relax the aortic strips precontracted by NA and the degree of the SOD-induced relaxation was significantly greater in diabetic aorta than in age-matched control vessels. From these results, it is most likely that the activity of SOD in the aorta is decreased in the diabetic state. Indeed, it has been found that the activity of SOD in various tissues is decreased in the diabetic state (Crouch *et al.*, 1978; Loven *et al.*, 1982;

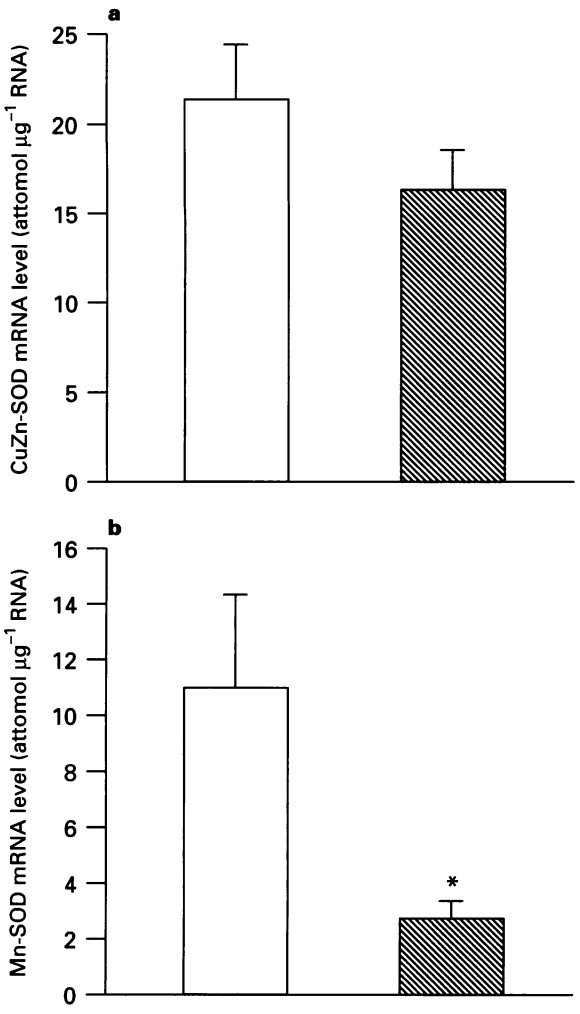

Figure 5 Changes in (a) Cu-Zn-SOD mRNA and (b) Mn-SOD mRNA levels in age-matched control and STZ-induced diabetic rats by competitive PCR. Total RNA was extracted as described in Methods. Each total RNA preparation (4.0 μg) was reverse transcribed and 10% portions were amplified in the presence of 2 μl of MIMIC dilution (10, 5, 2.5, 1.25, 0.625, 0.3125 attomol μl^{-1}, for 25 cycles). Age-matched control rats ($n=4$, open columns); STZ-induced diabetic rats ($n=4$, hatched columns). Values are mean $\pm$ s.e. of 4 determinations (attomol μg^{-1} RNA). Significantly different from age-matched control, $^*P<0.05$.

Matkovics *et al.*, 1982; Cohen, 1995). On the other hand, it has been shown that hydrogen peroxide accelerates NO release (Rubanyi & Vanhoutte, 1986b), activates soluble guanylate cyclase activity, and causes smooth muscle relaxation (Zembowicz *et al.*, 1993). Thus, an increase in SOD-induced relaxation in the diabetic state may be due to the overproduction of hydrogen peroxide which may cause smooth muscle relaxation, because diabetes causes a greater production of superoxide anion presumably due to reduced superoxide dismutase activity. However this is not the case because SOD-induced relaxation was not changed by preincubation with catalase.

Pretreatment with indomethacin did not prevent the rapid fade of the endothelium-dependent relaxation in tissues from the diabetic rats, indicating that constrictor prostanoids are not involved in the destruction of NO.

Superoxide anions (O_2^-) are oxygen radicals produced during mitochondria respiration (Boveris, 1977). To defend against the toxic effects of this free radical, cells have evolved an antioxidant enzyme system including SOD (Fridovich, 1974; Asayama *et al.*, 1985). These enzymes catalyse the dismutation of O_2^- into hydrogen peroxide and oxygen. There are two intracellular forms of SOD: Cu-Zn-SODs constitutively expressed (Visner *et al.*, 1990; Hsu *et al.*, 1994) and distributed throughout the cytoplasm (Fridovich, 1974). In contrast, Mn-SOD expression is highly regulated (Asayama *et al.*, 1985; Harris *et al.*, 1991) and localized specifically to mitochondria (Weisigner & Frifovich, 1973; Boveris, 1977). In the present study, mRNA expression of Mn-SOD was significantly decreased and Cu-Zn-SOD was slightly decreased in diabetic aorta as compared with age-matched control vessels. The present study is the first to demonstrate directly the decrease in mRNA expression of Mn-SOD or Cu-Zn-SOD in diabetic animals. A decrease in mRNA expression of Mn-SOD or Cu-Zn-SOD may result in a decreased tissue content of SOD. This may be the reason why preincubation of aortic rings with SOD normalized the recovery phase of the relaxation of diabetic aorta after single administration of ACh, and SOD-induced relaxation was significantly greater in diabetic aorta. Mn-SOD, localized in the mitochondria matrix, has an essential function reducing the cellular concentraton of superoxide radicals during both normal oxidative metabolism and periods of oxidative stress. The important role of Mn-SOD is demonstrated by the large variety of factors which regulate its expression (development, differentiation, oxidative stress, xenobiotics, cytokines; as reviewed by Harris, 1992). Most of these factors induce some modifications of the intracellular levels of reactive oxygen species. Thus, a free radical scavenger approach to the treatment of endothelial dysfunction in diabetic patients may have some potential merits. Indeed, in diabetic animals, chronic treatment of STZ-induced diabetic rates with the antioxidant, vitamin E, effectively preserves the endothelium-dependent relaxation (Keegan *et al.*, 1995).

Recently, we have demonstrated that the endothelium-dependent relaxation of aortic rings from STZ-induced diabetic mice was significantly attenuated, and the chronic administration of cholesterol-lowering drugs, cholestyramine, reduced serum low density lipoprotein (LDL) levels and normalized the endothelium-dependent relaxation (Kamata *et al.*, 1996). These results strongly suggest that endothelial dysfunction in STZ-induced diabetic mice is due to increased low-density lipoprotein (LDL) cholesterol. Endothelium dysfunction is intimately involved in the pathogenesis of atherosclerosis (Ross, 1986; Steinberg *et al.*, 1989; Yasue *et al.*, 1990). The oxidative modification of LDL cholesterol by the endothelium is thought to be an important step in the alteration of various endothelium functions (Kugiyama *et al.*, 1990; Rajavashisth *et al.*, 1990) and the initiation of atherosclerosis (Steinberg *et al.*, 1984). In several *in vitro* models of LDL oxidation, addition of SOD has been shown to be protective (Beckman *et al.*, 1990; Heinecke *et al.*, 1993; Kawamura *et al.*, 1994), suggesting a potential involvement of the superoxide radical in the process *in vivo*. The reduced content of SOD in the aorta should effectively enhance the direct involvement of the superoxide radical in LDL oxidation. It is unclear at present, however, whether the increased LDL cholesterol in the diabetic state can impair the mRNA expression of SOD in the aorta.

In conclusion, we have demonstrated that the rapid destruction of NO by superoxide anions may occur in STZ-induced diabetic rats, and this may be due to a decrease in mRNA expression of Mn-SOD or Cu-Zn-SOD.

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