Effects of Superoxide Dismutase on the Acetylcholine-induced Relaxation Response in Cholesterol-fed and Streptozotocin-induced Diabetic Mice

Katsuo Kamata, Masato Nakajima and Makoto Sugiura

Department of Physiology and Morphology, Institute of Medicinal Chemistry, Hoshi University

Abstract

High concentration of acetylcholine (ACh) caused a rapid and long-lasting relaxation response in age-matched controls, whereas this response was significantly weaker in streptozotocin (STZ)-diabetic and cholesterol-fed mice. The levels of basal and ACh-stimulated cyclic GMP in the aorta was also significantly smaller in STZ-diabetic and cholesterol-fed mice. The attenuated relaxation responses to ACh in both STZ-diabetic and cholesterol-fed mice were ameliorated by the chronic administration of cholestyramine. A prior incubation of aortic strips with superoxide dismutase (SOD, 60 U/ml) improved the recovery phase of the relaxation of diabetic aorta after single administration of ACh, whereas SOD had no effects on ACh-induced relaxation of aortic strips from cholesterol-fed mice. These results suggest that superoxide anion may be responsible for an impairment of endothelium-dependent relaxation of aorta from STZ-induced diabetic mice. It is further suggested that impairment of endothelium-dependent relaxation in STZ-diabetic and cholesterol-fed mice may be caused by different mechanisms.

Key words: streptozotocin, cholesterol-fed, aorta, cyclic GMP, endothelium, relaxation.

Introduction

Impaired endothelium-dependent relaxations in atherosclerosis have been reported in the rabbit aorta (Habib et al., 1986; Verbeuren et al., 1986; Bossaller et al., 1987; Jayakody et al., 1987; Simon et al., 1993), monkey iliac artery (Freiman et al., 1986), pig coronary artery (Yamamoto et al., 1987; Shimokawa and Vanhoutte, 1989), as well as human coronary artery in vitro (Bossaller et al., 1987; Forsterman et al., 1988) and in vivo (Lunder et al., 1986). In contrast, there are few reports concerning the endothelium-dependent relaxation to ACh in cholesterol-fed mice. The impairment of endothelium-dependent relaxations is thought to play an important role in the pathogenesis of coronary spasm. Oxidative modification of low-density lipoprotein (LDL) cholesterol by the endothelium is thought to be an important step in...
the initiation of atherosclerosis (Steinbrecher et al., 1984; Quin et al., 1987; Berliner et al., 1990). Oxidized LDL cholesterol impairs endothelium-dependent relaxation in isolated arteries (Kugiyama et al., 1990; Rajavashisth et al., 1990; Simon et al., 1990; Jacob et al., 1990; Yokoyama et al., 1990; Witztum et al., 1991; Flavahan, 1992). This inhibitory effect, which is not shared with native LDL, is mediated by lysophosphatidylcholine (LPC) (Kugiyama et al., 1990; Yokoyama et al., 1990; Kugiyama et al., 1992; Flavahan, 1993; Sugiyama et al., 1994). 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 and Gross, 1988; Kamata, et al., 1989a, b; Abiru et al., 1993; Cohen, 1995; Poston and Taylor, 1995).

Nitric oxide (NO) has been proposed as the major form of the endothelium-derived relaxing factor and contributes to arterial vasodilation (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 and Vanhoutte, 1986; Mian and Martip, 1995). Indeed, Hattori et al. (1991) reported that an enhanced 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 (Tesfamariam and Cohen, 1992; Hattori et al., 1991; Langenstroer and Pieper, 1992; Pieper et al., 1992) and in normal arteries incubated in a medium containing an elevated concentration of glucose (Tesfamariam and Cohen, 1992; Taylor and Poston, 1995). 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.

In the present study, therefore, we have examined the effect of SOD on an impairment of endothelium-dependent relaxation in the aorta from STZ-induced diabetic and cholesterol-fed mice. We were especially interested in determining which model is sensitive to this agent.

Materials and Methods

Male ICR mice aged 5 weeks and weighing 27.8±1.4 g were housed under constant climatic conditions (temperature 21°-22°C, relative air humidity 50±5%). The diets and water were given ad libitum to all animals. 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 and Culture, Japan).

Experimental design

Mice were randomly divided into two groups. Control mice received a standard mouse
diet, and cholesterol-fed mice received a diet supplemented with 2% cholesterol (wt/wt) and 0.5% cholic acid (wt/wt). This feeding program was adhered to for ten weeks. The experiments were performed ten weeks after the feeding.

Eight- to ten-week-old male ICR mice received a single injection of STZ (200 mg/kg) in the tail vein in order to induce diabetes. Age-matched controls were injected with a similar volume of citrate buffer. STZ-induced diabetic mice were fed a normal diet. Food and water were given ad libitum to all animals. The experiments were performed ten weeks after the injection.

Cholesterol-fed and STZ-induced diabetic mice received saline, cholestyramine (300 mg/kg, p.o. daily for 10 weeks). We administered this drug at the start of cholesterol-feeding or STZ injection.

Measurement of isometric force

After ten weeks of dietary intervention or STZ injection, the age-matched control, cholesterol-fed mice, STZ-induced diabetic mice, and hypercholesterolemic and diabetic mice that had been administered drug were anesthetized with ether, a midline incision was made, and blood was obtained from the abdominal aorta to be used to estimate serum cholesterol and serum glucose levels. The blood was centrifuged at 3,000 rpm for 10 min at 4°C and the serum was isolated and stored at -80°C. After the bleeding the aorta was rapidly dissected and placed in ice-cold modified Krebs-Henseleit solution (KHS, composition in mM: NaCl, 118.0; KCl, 4.7; NaHCO3, 25.0; CaCl2, 1.8; NaH2PO4, 1.2; MgSO4, 1.2; dextrose, 11.0). Each aorta was separated from surrounding connective tissue and cut into rings (3 mm long). Special care was taken not to damage the endothelium. The rings were then suspended in organ bath chambers, between a clip and a force-displacement transducer (TB-611T, Nihon Kohden, Japan) by means of two stainless steel wires inserted into the lumen, under a resting tension of 1.5 g (preliminary determined to be optimum), to measure isometric force. The organ chamber was filled with 10 ml of KHS at 37°C and gassed with 95% O2-5% CO2. Following a 1 hr equilibration period, prostaglandin F2α (PGF2α) was added to the organ bath at a concentration high enough (10⁻⁶-3×10⁻⁴ M) to induce ring contraction. After the PGF2α-induced contraction reached a plateau, 10⁻⁵ M ACh was added to the organ bath to confirm the integrity of the endothelium. The mice aortic rings were completely relaxed at this concentration of ACh. The removal of endothelial cells by rubbing was confirmed by the fact that tonic contraction of the aortic ring by PGF2α was not affected by ACh. The effects of drugs were then tested. The tissue was allowed to relax and equilibrate for 40 min before the next application of drugs. Because the maximal contraction of aortic rings in response to PGF2α was slightly enhanced in cholesterol-fed mice, for the relaxation studies aortic rings were precontracted with an equieffective concentration of 10⁻⁶ to 3×10⁻⁴ M PGF2α so that the rings would register a development of tension of approximately 900 mmg from age-matched control, cholesterol-fed and STZ-induced diabetic mice. When the PGF2α-induced contraction reached a plateau level, relaxant agents were added in a cumulative manner.
Measurement of cyclic GMP

Basal concentrations of, or ACh-induced changes in levels of cyclic GMP were measured in a separate series of experiments. Endothelium-intact aortic strips were allowed to equilibrate in tubes that contained KHS, gassed with 95% O₂, 5% CO₂, at 37°C for 60 min. One minute after the addition of the ACh, tissues were frozen in liquid N₂, and then homogenized in 1 ml of 6% trichloroacetic acid, and centrifuged at 3,000 g for 10 min. The supernatants were extracted three times in three volumes of water-saturated ether, and were then stored at −80°C until assayed for cyclic GMP. Following succinylation, levels of cyclic GMP were determined with radioimmunoassay kits (Yamasa Cyclic GMP Assay Kit, Yamasa Corp., Choshi, Japan). The recovery rate of the cyclic GMP contents in each column was calculated by counting the radioactivity due to [³H] cyclic GMP using a liquid scintillation counter (Aloka, Tokyo, Japan), and the values for cyclic GMP contents obtained from the radioimmunoassay were corrected using this recovery rate. The recovery rate for the cyclic GMP content in each column was within the range 85 to 95%. The release of cyclic GMP induced by methoxamine is expressed as fmol/100 μl.

Measurement of serum cholesterol and glucose

Serum cholesterol levels were determined using a commercially available enzyme kit (Wako Chemical Company, Osaka, Japan). The concentration of glucose in serum was determined by the o-toluidine method (Dubowski, 1962).

Drugs

Cholestyramine, streptozotocin, A23187 and sodium nitroprusside were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Acetylcholine was purchased from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). Prostaglandin F₂α (PGF₂α) was purchased from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). PGF₂α, SNP and ACh were dissolved in 0.9% saline immediately before each experiment. Concentrations are expressed as the final concentration of each drug in the organ bath.

Statistics

Data are expressed as the mean±S.E. Statistical differences were determined by Dunnett’s test for multiple comparisons, after a one-way analysis of variance, a probability level of P<0.05 being regarded as significant.

Results

Relaxation in response to ACh in age-matched control, cholesterol-fed and STZ-induced diabetic mice aortas

When the PGF₂α (10⁻⁶ to 3×10⁻⁶ M)-induced contraction reached a plateau, ACh or A23187 was cumulatively added. In aortic rings from age-matched control mice, ACh (10⁻⁹–10⁻⁵ M) or A23187 (10⁻⁸–10⁻⁶ M) caused concentration-dependent relaxation. The relaxations caused by ACh or A23187 were significantly weaker in rings from cholesterol-fed and STZ-
induced mice (Fig. 1). To clarify the characteristics of the ACh-induced relaxation, we examined its time course. When the PGF$_2\alpha$ ($10^{-6}$ to $3\times10^{-6}$ M)-induced contraction reached a plateau, single concentration of ACh ($10^{-6}$ M) was added. In aortic rings from age-matched control mice, ACh caused a rapid and long-lasting relaxation. The relaxations caused by ACh was significantly weaker in rings from cholesterol-fed and STZ-induced diabetic mice (Fig. 2). Treating the control mice with cholestyramine had no significant effect on the relaxation

![Figure 1](image-url)

**Fig. 1.** Concentration-response curves for ACh- and A23187-induced relaxation of aortic rings from age-matched control, cholesterol-fed and STZ-induced diabetic mice. The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by PGF$_2\alpha$ ($10^{-6}$ to $3\times10^{-6}$ M). Each point represents the mean±S.E.M. of 5 experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs. controls.
caused by ACh (data not shown). ACh-induced relaxation was significantly attenuated in STZ-induced diabetic mice, and the attenuated relaxation response of the aorta from STZ-induced diabetic mice was restored by the chronic administration of cholestyramine (300 mg/kg, p.o. daily for 10 weeks) as shown in Fig. 3. The relaxation caused by SNP (10⁻⁶ M) was not significantly different in aortic rings from the different groups (data not shown).

Changes in cyclic GMP levels in age-matched control, cholesterol-fed and STZ-induced diabetic mice aortas

Levels of cyclic GMP in aortic strips with endothelium from age-matched control, STZ-diabetic and cholesterol-fed mice were measured. Basal levels of cyclic GMP and ACh (10⁻⁶ M)-induced production of cyclic GMP were markedly lower in STZ-diabetic and cholesterol-fed mice (Fig. 4). In contrast, there were no differences in the formation of cyclic GMP stimulated by sodium nitroprusside (SNP) (10⁻⁶ M) among age-matched control, STZ-diabetic and cholesterol-fed mice.

Effects of superoxide dismutase or allopurinol on the ACh-induced relaxation

In control aortic strips, ACh (10⁻⁶ M)-induced long-lasting relaxation was not affected by SOD (60 U/ml). The attenuated relaxation response of aortic strips to ACh (10⁻⁶ M) seen in
Fig. 3. Effects of cholestyramine on ACh (10^-6 M)-induced relaxation in PGF_2alpha (10^-6 to 3x10^-6 M)-precontracted aortic rings from STZ-induced diabetic mice or STZ-diabetic mice treated with cholestyramine (300 mg/kg, p.o. daily for 10 weeks). The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by PGF_2alpha (10^-6 to 3x10^-6 M). Each point represents the mean±S.E.M. of 6 experiments. *P<0.05, **P<0.01, ***P<0.001 vs. controls.

STZ-diabetic mice was significantly restored by SOD (60 U/ml), whereas SOD could not improve the decreased relaxation to ACh in cholesterol-fed mice (Fig. 5).

Discussion

In the present study, we found that the chronic administration of cholestyramine preserved the endothelium-dependent relaxation of isolated aorta from cholesterol-fed and STZ-induced diabetic mice and that SOD restored an impaired endothelium-dependent relaxation in STZ-induced diabetic mice but not cholesterol-fed mice.

A reduction in the release of endothelium-derived relaxing factor (EDRF) from the vascular endothelium or a decrease in endothelium-dependent relaxation has been demonstrated in vascular tissues obtained from cholesterol-fed rabbits and in human atherosclerotic coronary arteries (Freiman et al., 1986; Habib et al., 1986; Verbeuren et al., 1986; Bossaller et al., 1987; Jayakody et al., 1987; Yamamoto et al., 1987; Forsterman et al., 1988; Shimokawa and Vanhoutte, 1989; Simon et al., 1993). Impaired endothelium-dependent relaxation has been observed in blood vessels of, STZ-induced diabetic rats (Oyama et al., 1986; Pieper and Gross, 1988; Kamata et al., 1989a, b, 1992; Abiru et al., 1993; Poston and Taylor, 1995) and alloxan-induced diabetic rabbits (Tesfamariam et al., 1989; Abiru et al., 1990a, b, 1991). Consistent with these findings, we found that ACh-induced endothelium-dependent relaxation
was significantly attenuated in both STZ-diabetic and cholesterol-fed mice. Data for an impaired endothelium-dependent relaxation were supported by the finding that basal levels of cyclic GMP and ACh-induced production of cyclic GMP were markedly lower in STZ-diabetic and cholesterol-fed mice. The impaired endothelium-dependent relaxation seen in STZ-induced diabetic rats has been said to be due to (1) decreased influx of Ca2+ into the endothelium (Kamata and Nakajima, 1998), (2) intimal cell proliferation and lipid deposition (Lopez et al., 1989), (3) L-arginine depletion (Egashira et al., 1996; Pieper, 1998), (4) an altered endothelial cell receptor-coupling mechanism (Cohen et al., 1988), (5) enhanced NO inactivation by superoxide anions (Gryglewski et al., 1986; Rubanyi and Vanhoutte, 1986; Pieper and Gross, 1988; Hattori et al., 1991; Langenstroer and Pieper, 1992; Kamata and Kobayashi, 1996), (6) oxidized LDL (Kugiyama et al., 1990; Chin et al., 1992; Ohara et al., 1993; Kobayashi and Kamata, 1998) or (7) hypertriglyceridaemia (Abe et al., 1998).

Cholestyramine is the chloride salt of a basic anion-exchange resin. Cholestyramine binds bile acids in the intestine, and there is a large increase in the fecal excretion of the acids. Cholestyramine increased the activity of 7-α-hydroxylase being the rate-limiting enzyme in
Fig. 5. Effects of superoxide dismutase (SOD, 60 U/ml) on ACh (10^{-6} M)-induced relaxation in PGF_2\alpha (10^{-6} to 3\times10^{-6} M)-precontracted aortic rings from control, STZ-diabetic or cholesterol-fed mice. The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by PGF_2\alpha (10^{-6} to 3\times10^{-6} M). Each point represents the mean±S.E.M. of 6 experiments. **P<0.01 diabetic control vs. diabetic mice treated with SOD.

bile acid formation (Grundy et al., 1971). These findings suggest that cholestyramine, by stimulating 7-\alpha-hydroxylase activity, and resultant bile acid synthesis, together with enhancing LDL receptor binding may affect the development of atherosclerosis (Shepherd et al., 1980; Kovanen et al., 1981). The endothelium-dependent relaxation of aortic rings in response to ACh was significantly attenuated in STZ-induced diabetic mice, and the impaired endothelium-dependent relaxation was restored by chronic administration of cholestyramine. These results suggest that endothelial dysfunction in STZ-induced diabetic mice is due to the increased LDL,
and that the endothelium-dependent relaxation may be preserved by the chronic administration of cholestyramine, at least in part, through lowering the serum LDL levels. Recently, we have reported that not only increased LDL cholesterol but also decreased activity of superoxide dismutase are responsible for the decreased relaxation response induced by acetylcholine (Kobayashi and Kamata, 1999).

Oxygen radicals, which inactivate endothelium-derived relaxing factor, have been implicated in impaired endothelium-dependent relaxation of the blood vessels from STZ-induced diabetic rats (Hattori et al., 1991; Langenstroer and Pieper, 1992; Pieper et al., 1993; Kamata and Kobayashi, 1996). Indeed, we have demonstrated that the expression of Mn$^{2+}$-superoxide dismutase mRNA was found to be significantly decreased in the aortas of 10-week diabetic rats (Kobayashi and Kamata, 1999). In that study, furthermore, the activity of superoxide dismutase was also significantly decreased in 10-week diabetic rats. These results can be explained if the reduction in the content of superoxide dismutase in the diabetic aorta were to reduce the inactivation of the superoxide anion: the resultant increase in the superoxide radical would increase the oxidation of LDL or metabolize the NO to NO$_2^-$ or NO$_3^-$. In the aortic rings from STZ-diabetic rats, the endothelium-dependent relaxation was restored by SOD, indicating that the released NO from the endothelium may be metabolized by superoxide anions. This may be the reason why SOD was effectively ameliorated the ACh-induced relaxation. On the other hand, in the aortic rings from cholesterol-fed mice, the decreased relaxation response to ACh was not restored by SOD. These results suggest that involvement of superoxide anion in the impairment of the endothelium-dependent relaxation may differ between diabetes and hypercholesterolemia. Further studies are required on this point.

Since A23187-induced relaxations were also reduced in both cholesterol-fed and STZ-induced diabetic mice, the impairment of endothelium-dependent relaxation by high levels of cholesterol is not due to muscarinic antagonistic action.

In conclusion, superoxide anion may be responsible for an impairment of endothelium-dependent relaxation of aorta from STZ-induced diabetic mice. It is further suggested that impairment of endothelium-dependent relaxation in STZ-diabetic and cholesterol-fed mice may be caused by different mechanisms.

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