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Preservation of endothelium-dependent relaxation in cholesterol-fed and streptozotocin-induced diabetic mice by the chronic administration of cholestyramine

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1 Experiments were designed to investigate the effects of the low density lipoprotein (LDL)-lowering drugs cholestyramine on serum LDL levels and endothelium-dependent relaxation to acetylcholine (ACh) in cholesterol-fed or streptozotocin (STZ)-induced diabetic mice.

2 In aortic rings from control mice, ACh or A23187 caused concentration-dependent relaxation. The relaxations caused by ACh or A23187 were significantly attenuated in aortic rings from cholesterol-fed and STZ-diabetic mice. The attenuated vasodilatation in both cholesterol-fed and diabetic mice was returned to normal by chronic administration of cholestyramine. The endothelium-independent relaxations of aortic rings induced by sodium nitroprusside (SNP) were not significantly different between control, cholesterol-fed and STZ-induced diabetic mice.

3 The increased LDL levels in cholesterol-fed and diabetic mice were returned to normal by the chronic administration of cholestyramine. Chronic administration of cholestyramine had no effects on serum glucose levels.

4 These results suggest that attenuated endothelium-dependent vasodilatations in both cholesterol-fed and STZ-diabetic mice are improved by the chronic administration of cholestyramine, and these effects are, at least in part, due to lowering serum LDL levels.

Keywords: Endothelium; LDL; cholesterolaemia; A23187; streptozotocin; cholestyramine; diabetic mouse

Introduction

Impaired endothelium-dependent relaxations in atherosclerosis have been reported in the rabbit aorta (Habib *et al.*, 1987; Verbeuren *et al.*, 1986; Bossaller *et al.*, 1987; Joykody *et al.*, 1987; Simon *et al.*, 1993), monkey iliac artery (Freiman *et al.*, 1986), pig coronary artery (Yamamoto *et al.*, 1987; Shimokawa & Vanhoutte, 1989), as well as human coronary artery *in vitro* (Bossaller *et al.*, 1987; Fosterman *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) (Yokoyama *et al.*, 1990; Kugiyama *et al.*, 1990; 1992; Flavahan, 1993; Sugiyama *et al.*, 1994).

It is well known that vascular disease is one of the complicating features of diabetes mellitus in man (Christrieb, 1973). The reactivity of vascular smooth muscles and endothelium to vasoactive agents in diabetic animals has been extensively studied (Agrawal & McNeill, 1987; Harris & Macleod, 1988; Kamata *et al.*, 1992). It has been shown that relaxation response of aortic strips to endothelium-dependent agents was decreased in STZ-induced diabetic rats (Oyama *et al.*, 1986; Pieper & Gross, 1988; Kamata *et al.*, 1989a,b; Taylor *et al.*, 1992; 1994a,b; Abiru *et al.*, 1993; Poston & Taylor, 1995) and alloxan-induced diabetic rabbits (Tesfamariam *et al.*, 1989; Abiru *et al.*, 1990a,b, 1991). There are few studies which have directly assessed the role of hyperlipidaemia or oxidized LDL in endothelial cells dysfunction in diabetes. To our knowledge, no studies have investigated the effect of cholesterol-lowering therapy on endothelial function in animal models of diabetes or in man.

Data on the preservation of endothelium-dependent relaxation in cholesterol-fed and STZ-induced diabetic animals by chronic administration of cholestyramine are lacking.

The purpose of the present study was to define the relationship between serum LDL levels and endothelial dysfunction in cholesterol-fed and STZ-induced diabetic mice.

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%). Food and water was 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 programme was adhered to for 10 weeks. The experiments were performed 10 weeks after the feeding.

Eight- to ten week-old male ICR mice received a single injection of STZ (200 mg kg^{-1}) in the tail vein in order to induce

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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 10 weeks after the injection.

Cholesterol-fed and STZ-induced diabetic mice received saline, cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks). We administered this drug at the start of cholesterol-feeding or STZ injection.

Measurement of isometric force

After 10 weeks of dietary intervention of STZ injection, the age-matched control, cholesterol-fed mice, STZ-induced diabetic mice, and hypercholesterolaemic and diabetic mice that had been administered drug were anaesthetized 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 3000 r.p.m. 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, $NaHCO_3$ 25.0, $CaCl_2$ 1.8, NaH_2PO_4 1.2; $MgSO_4$ 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% O_2:5% CO_2. Following a 1 h equilibration period, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was added to the organ bath at a concentration high enough ($10^{-6}-3\times10^{-6}$ M) to induce ring contraction. After the $PGF_{2\alpha}$-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 $PGF_{2\alpha}$ 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 $PGF_{2\alpha}$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^{-6} M $PGF_{2\alpha}$ so that the rings would register a development of tension of approximately 900 mg from age-matched control, cholesterol-fed and STZ-induced diabetic mice. When the $PGF_{2\alpha}$-induced contraction reached a plateau level, relaxant agents were added in a cumulative manner.

Measurement of serum cholesterol and glucose

Serum cholesterol levels were determined with 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, U.S.A.). Acetylcholine was purchased from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was purchased from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). $PGF_{2\alpha}$, 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 means ± s.e.mean. Statistical differences were measured by Student's *t* test for unpaired observations, following one-way analysis of variance.

Results

Relaxation in response to ACh, A23187 or SNP in age-matched control, cholesterol-fed and STZ-induced diabetic mice aortae

When the $PGF_{2\alpha}$ (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 ($10^{-9}-10^{\times5}$ M) or A23187 ($10^{-8}-10^{-6}$ M) caused concentration-dependent relaxation. The relaxations caused by ACh or A23187 were significantly decreased in rings from cholesterol-fed mice (Figures 1 and 2). After chronic administration of cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks), aortic rings from cholesterol-fed mice relaxed in a normal response to ACh (Figures 1 and 2). Treatment of the control mice with cholestyramine had no significant effect on the relaxation caused by ACh (data not shown). Similarly, ACh- or A23187-induced relaxations were significantly attenuated in STZ-induced diabetic mice, and the attenuated relaxation responses of the aortae from STZ-induced diabetic mice were returned to normal by the chronic administration of cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks) as shown in Figures 3 and 4. The relaxation caused by SNP ($10^{-9}-10^{-5}$ M) was not significantly different in aortic rings from the different groups (Figures 5 and 6). Treatment of control mice with cholestyramine had no significant effect on relaxation caused by SNP (data not shown).

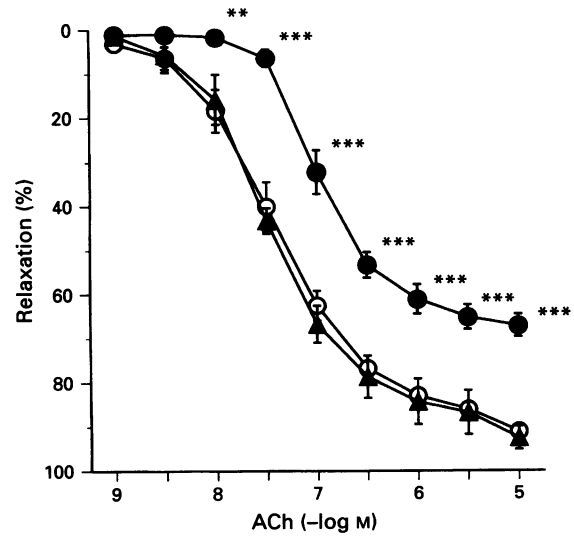

Figure 1 Concentration-response curves for ACh-induced relaxation of aortic rings obtained from age-matched control mice, cholesterol-fed mice and cholesterol-fed mice given cholestyramine. The aortic rings were initially contracted with $PGF_{2\alpha}$ ($10^{-6}-3\times10^{-6}$ M). Age-matched control mice ($n=6$, ○); cholesterol-fed mice ($n=6$, ●); cholesterol-fed, cholestyramine-treated mice ($n=6$, ▲). The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by $PGF_{2\alpha}$($10^{-6}-3\times10^{-6}$ M). Each data point on the graph represents the mean ± s.e. of six experiments; the s.e. are only included when they exceed the dimension of the symbols used. $^{**}P<0.01$, $^{***}P<0.001$.

Effects of chronic administration of cholestyramine on serum cholesterol and glucose levels in cholesterol-fed and STZ-induced diabetic mice

In cholesterol-fed and STZ-induced diabetic mice, serum total cholesterol and LDL cholesterol levels were significantly increased (Figures 7 and 8). Chronic administration of cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks) significantly reduced the total cholesterol and LDL cholesterol levels in cholesterol-fed and STZ-induced diabetic mice as shown in Figures 7 and 8. Serum glucose levels were not different between age-matched control mice, cholesterol-fed mice and cholesterol-fed mice that received cholestyramine (Figure 7). The increased levels of serum glucose in STZ-induced diabetic mice were not affected by the chronic administration of cholestyramine (Figure 8).

Discussion

In the present study, we found that the chronic administration of cholestyramine preserves the endothelium-dependent relaxation of isolated aortae from cholesterol-fed and STZ-induced diabetic mice and significantly reduces the total cholesterol and LDL cholesterol levels in cholesterol-fed and STZ-induced diabetic 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; Joykody *et al.*, 1987; Yamamoto *et al.*, 1987; Forsterman *et al.*,1988; Shimokawa & Vanhoutte, 1989; Simon *et al.*, 1993). Consistent with these findings, we found that ACh-induced endothelium-dependent relaxation was significantly attenuated in cholesterol-fed mice. Since the endothelium-independent relaxation of mouse aortic rings by SNP was not changed in cholesterol-fed mice, activity of soluble guanylate cyclase in the smooth muscle of the aorta was not altered in cholesterol-fed mice. These results suggest that the mouse is also useful as an animal model of hypercholesterolaemia.

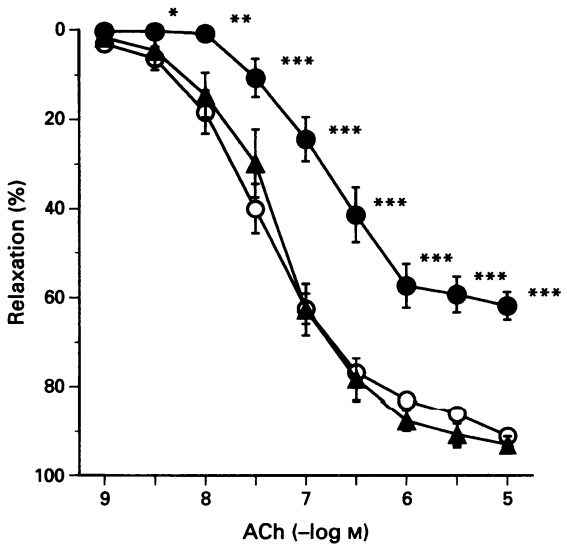

Figure 3 Concentration-response curves for ACh-induced relaxation of aortic rings obtained from age-matched control mice, STZ-induced mice and STZ-induced mice given cholestyramine. The aortic rings were initially contracted with $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Age-matched control mice ($n=6$, ○); STZ-induced diabetic mice ($n=6$, ●); STZ-induced diabetic, cholestyramine-treated mice ($n=6$, ▲). The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Each data point on the graph represents the mean±s.e. of six experiments; the s.e. are included only when they exceed the dimension of the symbols used. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

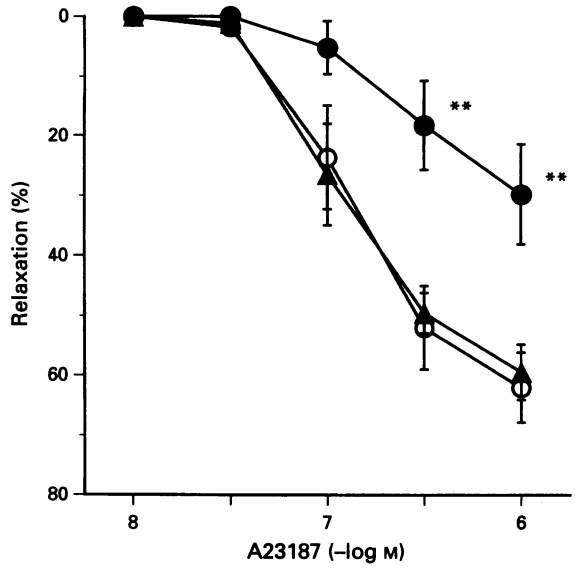

Figure 2 Concentration-response curves for A23187-induced relaxation of aortic rings obtained from age-matched control mice, cholesterol-fed mice and cholesterol-fed mice given cholestyramine. The aortic rings were initially contracted with $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Age matched control mice ($n=6$, ○); cholesterol-fed mice ($n=6$, ●); cholesterol-fed, cholestyramine-treated mice ($n=6$, ▲). The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Each data point on the graph represents the mean±s.e. of six experiments; the s.e. are included only when they exceed the dimension of the symbols used. $^{**}P<0.01$.

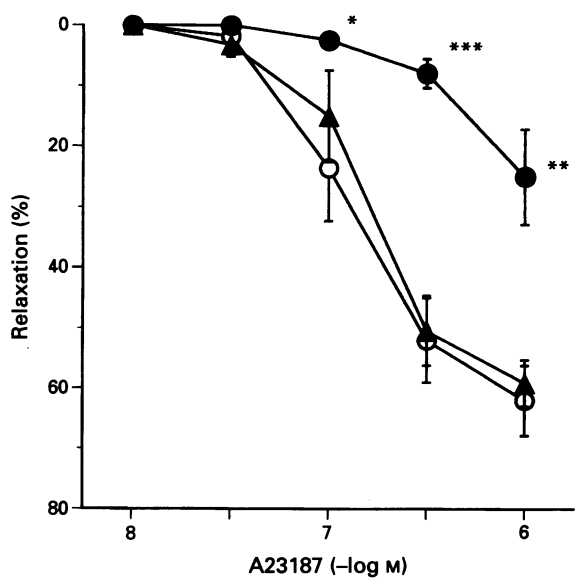

Figure 4 Concentration-response curves for A23187-induced relaxation of aortic rings obtained from age-matched control mice, STZ-induced mice and STZ-induced mice given cholestyramine. The aortic rings were initially contracted with $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Age-matched control mice ($n=6$, ○); STZ-induced diabetic mice ($n=6$, ●); STZ-induced diabetic, cholestyramine-treated mice ($n=6$, ▲). The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Each data point on the graph represents the mean±s.e. of six experiments; the s.e. are included only when they exceed the dimension of the symbols used. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

Impaired endothelium-dependent relaxation has been observed in blood vessels of genetically diabetic rats (Durante *et al.*, 1988; Meraji *et al.*, 1989; Miyata *et al.*, 1992; 1993), STZ-induced diabetic rats (Oyama *et al.*, 1986; Pieper & Gross, 1988; Kamata *et al.*, 1989a,b, 1992; Taylor *et al.*, 1992, 1994a,b; Abiru *et al.*,1993; Poston & Taylor, 1995) and alloxan-induced diabetic rabbits (Tesfamariam *et al.*, 1989; Abiru *et al.*, 1990a,b; 1991). In the present study, we used the mouse as an animal model of diabetes, and have shown that ACh-induced endothelium-dependent relaxation was reduced in STZ-induced diabetic mice, suggesting that the mouse is also useful as an animal model of diabetes.

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 faecal excretion of the acids. Cholestyramine increased the activity of 7-α-hydroxylase, the latter being the rate-limiting enzyme in bile acid formation (Grundy *et al.*, 1971). These findings suggest that cholestyramine, by stimulating 7-α-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). Although cholesterol-

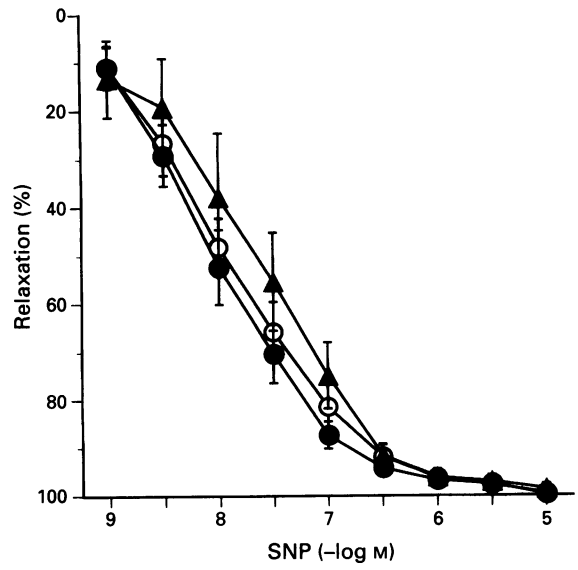

Figure 5 Concentration-response curves for sodium nitroprusside (SNP)-induced relaxation of aortic rings obtained from age-matched control mice, cholesterol-fed mice and cholesterol-fed mice given cholestyramine. The aortic rings were initially contracted with $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Age-matched control mice ($n=6$, ○); cholesterol-fed mice ($n=6$, ●); cholesterol-fed, cholestyramine-treated mice ($n=6$, ▲). The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Each data point on the graph represents the mean±s.e. of six experiments; the s.e. are included only when they exceed the dimension of the symbols used.

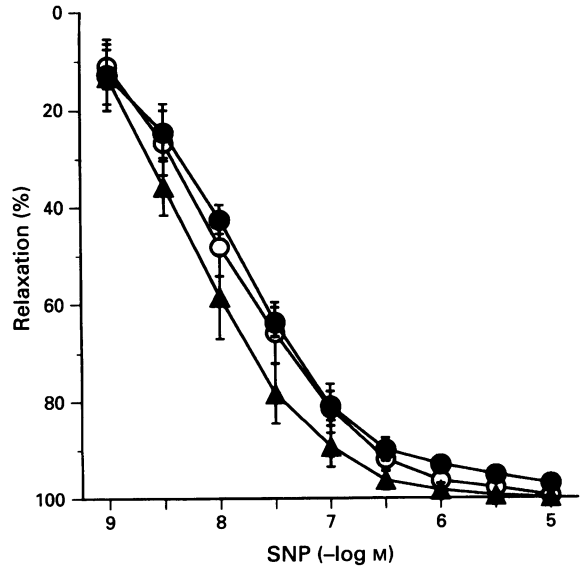

Figure 6 Concentration-response curves for sodium nitroprusside (SNP)-induced relaxation of aortic rings obtained from age-matched control mice, STZ-induced mice and STZ-induced mice given cholestyramine. The aortic rings were initially contracted with $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Age-matched control mice ($n=6$, ○); STZ-induced diabetic mice ($n=6$, ●); STZ-induced diabetic, cholestyramine-treated mice ($n=6$, ▲). The ordinate scale represents the relaxation of aortic rings as a percentage of the contraction induced by $PGF_{2\alpha}$ (10^{-6}–3×10^{-6} M). Each data point on the graph represents the mean±s.e. of six experiments; the s.e. are included only when they exceed the dimension of the symbols used.

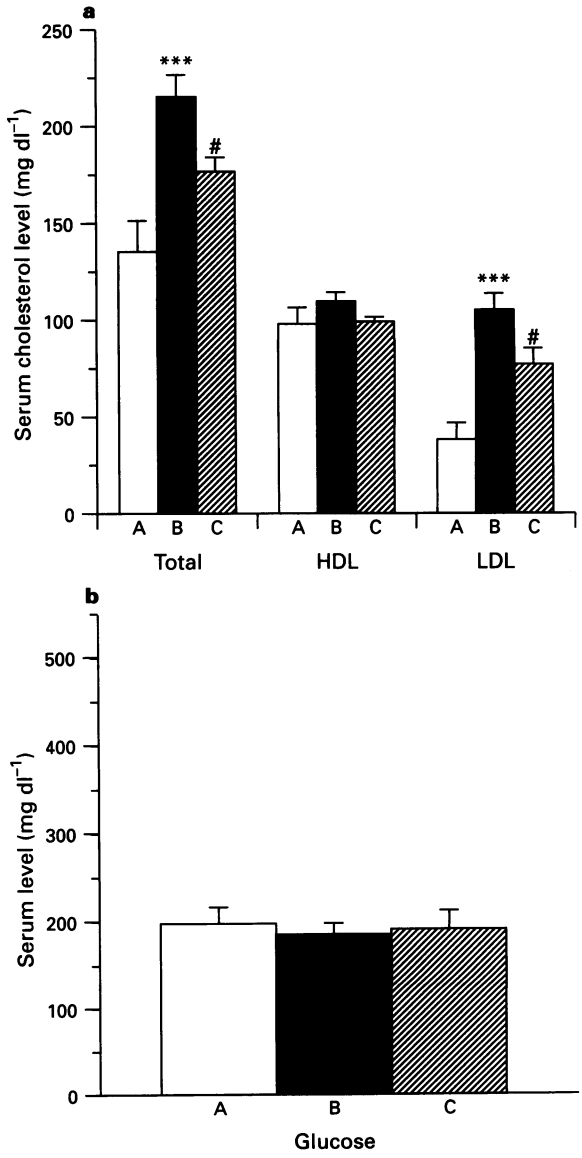

Figure 7 Effects of cholestyramine on levels of cholesterol and glucose in cholesterol-fed mice. Cholesterol-fed mice received cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks). (A) ($n=6$) age-matched control mice; (B) ($n=6$) cholesterol-fed mice; (C) ($n=6$) cholesterol-fed, cholestyramine-treated mice. $^{***}P<0.001$, cholesterol vs. control, $^{\#}P<0.05$, cholesterol-fed group vs. cholesterol-fed mice receiving drug.

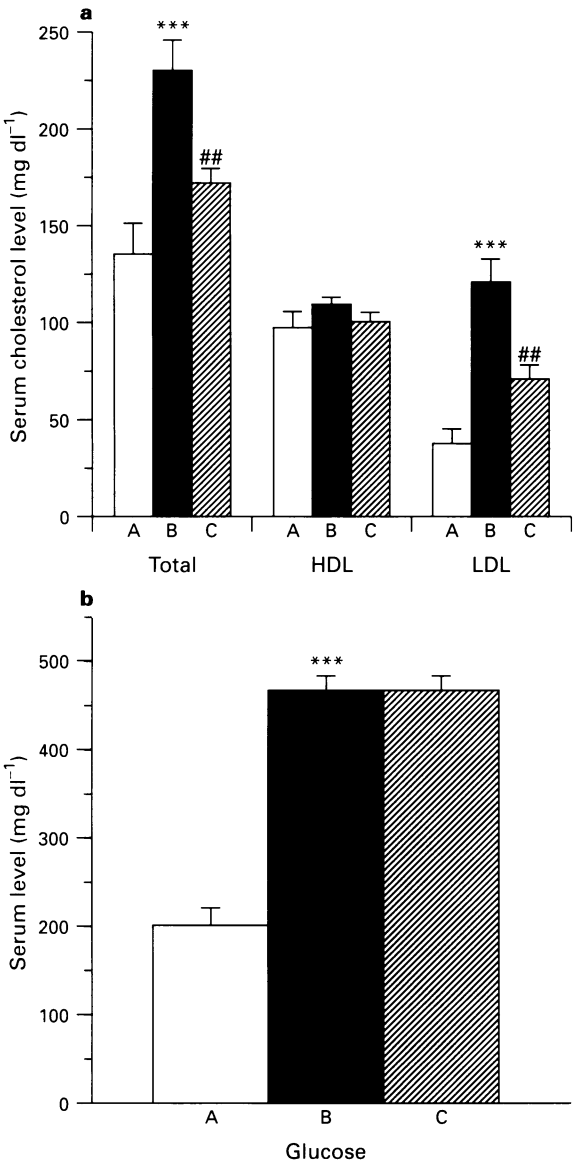

Figure 8 Effects of cholestyramine on levels of cholesterol and glucose in STZ-induced diabetic mice. STZ-induced mice received cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks). (A) ($n=6$) age-matched control mice; (B) ($n=6$) STZ-induced diabetic mice; (C) ($n=6$) STZ-induced diabetic, cholestyramine-treated mice. $^{***}P<0.001$, diabetic vs. control, $^{\#\#}P<0.01$, STZ-diabetic group vs. STZ-diabetic mice receiving drug.

lowering drugs have a beneficial effect on lipids, there are few reports concerning the positive effects of these agents on endothelial dysfunction in cholesterolaemia or diabetic states after the chronic administration of these agents in animals. In cholesterol-fed and STZ-induced diabetic mice, serum total cholesterol and LDL cholesterol levels were significantly increased and the increased cholesterol levels were normalized by the chronic administration of cholestyramine. The endothelium-dependent relaxation of aortic rings in response to ACh was significantly attenuated in cholesterol-fed and STZ-induced diabetic mice and the impaired endothelium-dependent relaxation was restored by chronic administration of cholestyramine. These results suggest that endothelial dysfunction in cholesterol-fed and 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.

An endothelium dysfunction is intimately involved in the pathogenesis of atherosclerosis (Ross, 1986; DiCorleto & Chisolm, 1986; Bossaller *et al.*, 1987; 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; Witztum & Steinberg, 1984; Flavahan, 1992) and the initiation of atherosclerosis (Steinberg *et al.*, 1984). LPC, which is transferred from oxidized LDL to the endothelial surface membrane, is involved in the mechanisms of the endothelial functional alterations caused by oxidized LDL (Kugiyama *et al.*, 1990;1992; Flavahan, 1993; Sugiyama *et al.*, 1994). Indeed, we confirmed that the endotheliun-dependent relaxation of aortic strips in response to ACh was significantly attenuated by pretreatment with LPC (Kamata *et al.*, 1989b). Therefore, the release of LPC from oxidized LDL must play an important role in endothelial function. If this were the case, the following sequence of events would be predicted in cholesterol-fed and STZ-induced diabetic mice: serum LDL levels are increased in cholesterol-fed and STZ-induced diabetic mice; increased LDL is oxidized on the endothelium; LPC is transferred from oxidized LDL; LPC may inhibit the endothelium-dependent vasodilatation induced by agonists, thereby resulting in endothelial dysfunction in cholesterol-fed and STZ-induced diabetic mice. In the present study, the chronic administration of cholestyramine significantly lowered serum LDL levels. This cholesterol-lowering effects of cholestyramine may improve the endothelium-dependent relaxation.

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; Langenstoer & Pieper, 1992; Pieper *et al.*, 1993). Indeed, Pieper *et al.* (1992) have demonstrated, using a bioassay technique for endothelium-derived relaxing factor, that free radicals mediate the destruction of nitric oxide (NO) in diabetic rat aorta. It is unclear at present whether increased LDL in the diabetic state can produce superoxide anions in the aorta; this requires further investigation.

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, we have demonstrated that the endothelium-dependent relaxation of aortic rings from cholesterol-fed or STZ-induced diabetic mice was significantly attenuated. The chronic administration of cholestyramine reduced serum LDL levels and restored the endothelium-dependent relaxation.

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