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Ca^{2+} mobilization in the aortic endothelium in streptozotocin-induced diabetic and cholesterol-fed mice

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1 Experiments were performed to compare Ca^{2+} mobilization in the aortic endothelium in streptozotocin (STZ)-induced diabetic and cholesterol-fed mice with that in age-matched controls.

2 The intracellular free Ca^{2+} ($[Ca^{2+}]_i$) in the fura PE-3 loaded endothelium of aortic rings was dose-dependently increased by cumulative administration of acetylcholine (ACh). ACh caused a transient rise in $[Ca^{2+}]_i$ in Ca^{2+}-free medium. The ACh-induced increase in $[Ca^{2+}]_i$ in normal or Ca^{2+}-free medium was significantly weaker in both STZ-induced diabetic and cholesterol-fed mice.

3 The weaker $[Ca^{2+}]_i$ response in Ca^{2+}-containing medium in STZ-induced diabetic and cholesterol-fed mice was normalized by chronic administration of cholestyramine.

4 The increased low density lipoprotein (LDL) levels seen in both STZ-induced diabetic and cholesterol-fed mice were normalized by the same chronic administration of cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks). Chronic administration of cholestyramine had no effect on the plasma glucose level.

5 Lysophosphatidylcholine (LPC) decreased the $[Ca^{2+}]_i$ responses to ACh in the aortic endothelium from normal mice.

6 These results suggest that ACh increases both Ca^{2+} influx and Ca^{2+} release from storage in the aortic endothelium. The weaker $[Ca^{2+}]_i$ influx seen in the endothelium of aortae from both STZ-induced diabetic and cholesterol-fed mice was improved by the chronic administration of cholestyramine, and we suggest that this improvement is due, at least in part, to a lowering of the plasma LDL level. It is further suggested that LPC may have an important influence over Ca^{2+} mobilization in the endothelium.

Keywords: Ca^{2+}; endothelium; nitric oxide; diabetes; cholesterolaemia; aorta

Introduction

It has been shown that the relaxation response of aortic strips to endothelium-dependent agents is decreased both in STZ-induced diabetic rats (Oyama *et al*., 1986; Pieper & Gross, 1988; Kamata *et al*., 1989a,b; Abiru *et al*., 1993; Cohen, 1993; Poston & Taylor, 1995) and in alloxan-induced diabetic rabbits (Tesfamariam *et al*., 1989; Abiru *et al*., 1990a,b; 1991).

Impairment of endothelium-dependent relaxations is thought to play an important role in the pathogenesis of coronary spasm. Further, 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 & Steinber, 1991; Flavahan, 1992). This inhibitory effect, which is not shared with native LDL, is mediated by lysophosphatidylcholine (LPC) (Kugiyama *et al*., 1990; 1992; Yokoyama *et al*., 1990; Flavahan, 1993; Sugiyama *et al*., 1994).

We recently showed that the attenuated endothelium-dependent relaxation seen in both cholesterol-fed and STZ-diabetic mice can be improved by the chronic administration of cholestyramine, a cholesterol-lowering drug (Kamata *et al*., 1996). However, the mechanisms underlying the impairment effects exerted by LDL and LPC, which is released from oxidized LDL, are unclear at present.

Endothelium-derived nitric oxide (NO), which is synthesized from L-arginine exerts strong vasodilator effects with an accompanying accumulation of guanosine 3′:5′-cyclic monophosphate (cyclic GMP) (Moncada *et al*., 1991). A common feature of endothelium-dependent vasodilators is their ability to elevate cytosolic Ca^{2+} in endothelial cells (Furchgott, 1984; Johns *et al*., 1987; Luckhoff *et al*., 1988). However, there have been no studies on the change in Ca^{2+} influx into the endothelial cells in vessels obtained from diabetic or cholesterol-fed mice. Furthermore, to our knowledge no studies have yet investigated the effect of cholesterol-lowering therapy on Ca^{2+} influx into the endothelium in animal models of diabetes or hypercholesterolaemia.

The main purpose of the present study was to determine whether Ca^{2+} influx into the endothelium is altered in mice with STZ-induced diabetes or hypercholesterolaemia. We also investigated the relationship between plasma LDL levels and Ca^{2+} influx into the endothelium in these same 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 $50 \pm 5\%$). The diets and water were given *ad libitum* to all animals. The studies were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals in Hoshi University, adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Science, Sports and Culture.

Experimental design

Mice were randomly divided into two groups. Control mice received a standard mouse diet while cholesterol-fed mice

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received a diet supplemented with 2% cholesterol (wt/wt) and 0.5% cholic acid (wt/wt). This feeding programme was adhered to for ten weeks. The experiments were performed at the end of this ten-week period.

Eight- to ten-week-old male ICR mice received a single injection of STZ (200 mg kg^{-1}) via the tail vein in order to induce diabetes. Age-matched controls were injected with a similar volume of vehicle (citrate buffer). STZ-induced diabetic mice were fed the same diet as the control mice. Food and water were given *ad libitum* to all animals. The experiments were performed ten weeks after the injection.

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

Preparation of aortic strips and measurement of intracellular free Ca^{2+}

After ten weeks of dietary intervention (ten weeks after the vehicle or STZ injection), the age-matched controls, cholesterol-fed mice, STZ-induced diabetic mice, and hypercholesterolaemic and diabetic mice that had been given cholestyramine or saline were anaesthetized with either. A midline incision was made and blood was obtained from the abdominal aorta to be used for the estimation of plasma cholesterol and plasma 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 had been completed, the aorta was rapidly dissected out 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 and dextrose 11.0). Each aorta was separated from the surrounding connective tissue and cut into rings (3 mm long). Special care was taken not to damage the endothelium. In some rings, the endothelium was removed by rubbing the intimal surface with a cotton swab. Effective removal was confirmed by the absence of a relaxing effect of 10 μM ACh during prostaglandin $F_{2\alpha}$-induced contraction.

$[Ca^{2+}]_i$ was measured by the method of Sato *et al.* (1988). Aortic ring preparations were exposed to 10^{-5} M fura PE3-AM in the presence of 0.04% cremophor EL for 5 h at 26°C. The tissue was then rinsed for 30 min and suspended in an organ bath containing KHS at 37°C. Fluorescence was measured with a fluorometer (Japan Spectroscopic, CAF 110, Tokyo, Japan) with excitation at 340 and 380 nm, and emission at 500 nm. The ratio (F_{340}/F_{380}) of the fluorescence signals was used to provide an index of $[Ca^{2+}]_i$. The tissue was equilibrated under a resting tension of 0.5 g. To enable examination of the effect of acetylcholine on Ca^{2+} release from the storage sites, some experiments were conducted in Ca^{2+}-free KHS containing 0.5 mM ethylene glycol-bis-(beta-aminoethyl ether) N,N,N′,N′,-tetra-acetic acid (EGTA). Ca^{2+}-free medium was prepared by replacing the $CaCl_2$ with NaCl.

Measurement of plasma cholesterol and glucose

Plasma cholesterol levels were determined by use of a commercially available enzyme kit (Wako Pure Chemical Co. Ltd., Osaka, Japan). The concentration of glucose in plasma was determined by the *o*-toluidine method (Dubowski, 1962).

Drugs

Streptozotocin, cremophor E, Lethylene glycol-bis-(beta-aminoethyl ether) N,N,N′,N′,-tetra-acetic acid (EGTA), and cholestyramine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Acetylcholine chloride was purchased from Daiichi Pharmaceutical Co. Ltd. (Tokyo, Japan). Prostaglandin $F_{2\alpha}$ was purchased from Ono Pharmaceutical Co. Ltd. (Osaka, Japan). Fura PE3-AM was purchased from Wako Pure Chemical Co. Ltd. (Osaka, Japan). Fura PE3-AM was dissolved in dimethylsulphoxide (DMSO) and mixed with cremophor EL. ACh was dissolved in 0.9% saline immediately before each experiment. Concentrations are expressed as the final concentration of the drug in the organ bath.

Statistics

Data are expressed as the mean ± s.e.mean. When comparisons were made between groups, Dunnet's multiple comparison test was used. Differences were considered statistically significant at the 5% level.

Results

Intracellular free Ca^{2+} in the endothelium of aortae from STZ-induced diabetic and cholesterol-fed mice

Typical changes in intracellular free Ca^{2+} ($[Ca^{2+}]_i$) evoked by ACh in fura-PE-3-loaded aortic rings are illustrated in Figure 1. ACh (10^{-7} to 10^{-5} M) evoked a dose-dependent increase in $[Ca^{2+}]_i$ in endothelium-intact aortic rings in normal KHS, but did not increase $[Ca^{2+}]_i$ at all in the endothelium-denuded rings ($n = 6$). Subsequent experiments were performed with endothelium-intact rings. The increase in $[Ca^{2+}]_i$ induced by ACh was significantly smaller in both STZ-induced diabetic and cholesterol-fed mice (Figure 2). The ACh-induced increase in $[Ca^{2+}]_i$ was not changed by incubating aortic rings with 44 mM glucose for 6 h. These data are summarized in Figure 3.

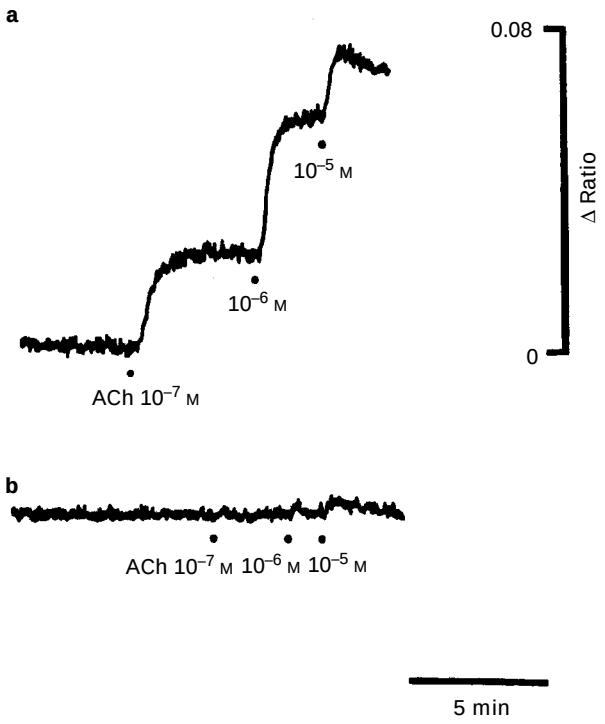

Figure 1 Typical traces showing intracellular Ca^{2+} in aortic rings with (a) or without (b) endothelium. The rings were obtained from control mice. Acetylcholine (10^{-7} to 10^{-5} M) was applied cumulatively as shown.

In Ca^{2+}-free medium, the ACh (10^{-5} M)-induced increase in $[Ca^{2+}]_i$ was transient, while ACh (10^{-5} M) produced a large and long-lasting increase in $[Ca^{2+}]_i$ in normal KHS (1.8 mM Ca^{2+}) (Figure 4). These responses can be assumed to represent Ca^{2+} release from the store sites and Ca^{2+} influx, respectively. Both the transient and the long-lasting increases in $[Ca^{2+}]_i$ evoked by ACh were significantly smaller in both STZ-induced diabetic and cholesterol-fed mice (Figure 5).

After chronic administration of cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks), the $[Ca^{2+}]_i$ response to ACh in Ca^{2+}-containing medium was restored to normal in both STZ-induced diabetic and cholesterol-fed mice (Figure 6). Chronic administration of cholestyramine (300 mg kg^{-1}, p.o. daily for 10 weeks) had no effect on the $[Ca^{2+}]_i$ response to ACh in control mice (data not shown).

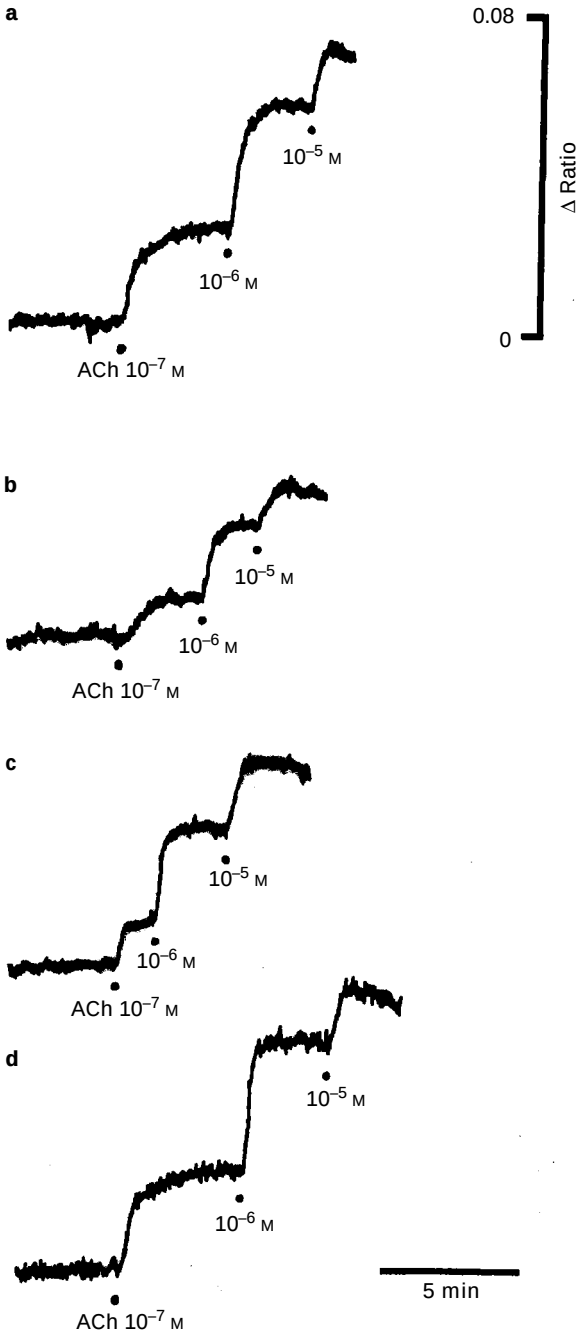

Figure 2 Typical traces showing intracellular Ca^{2+} in aortic rings with endothelium intact. The rings were obtained from (a) control, (b) STZ-induced diabetic and (c) cholesterol-fed mice. (d) The trace (from a control mouse) was obtained from a ring treated with 44 mM glucose for 6 h. Acetylcholine (10^{-7} to 10^{-5} M) was cumulatively applied as shown.

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

Chronic administration of cholestyramine (300 mg kg^{-1}, p.o., daily for 10 weeks) significantly lowered both total cholesterol

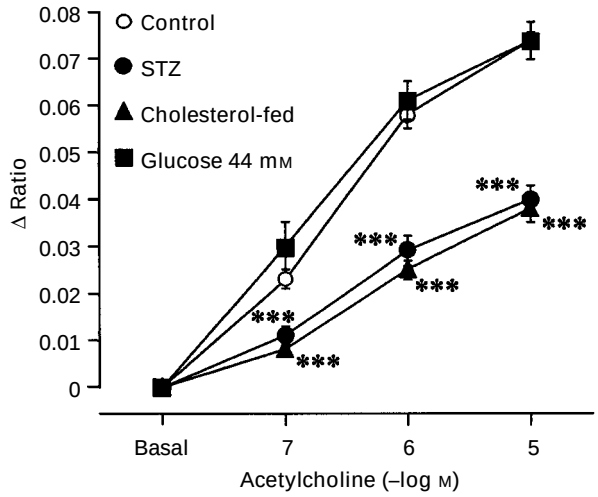

Figure 3 Dose-response curves for ACh-induced increase in intracellular Ca^{2+} in aortic rings from STZ-induced diabetic mice ($n=9$), cholesterol-fed mice ($n=11$) and control mice ($n=7$) treated or not treated with 44 mM glucose for 6 h ($n=6$). Each data point represents the mean and vertical lines show s.e.; the s.e. is shown only when it exceeds the dimension of the symbol used $^{***}P<0.001$ vs controls.

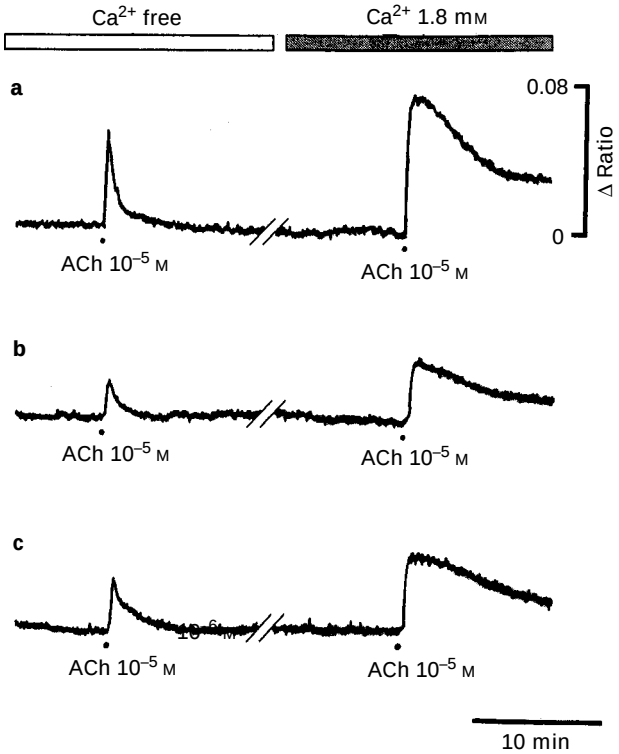

Figure 4 Typical traces showing evoked changes in intracellular Ca^{2+} in aortic rings in Ca^{2+}-free medium and normal KHS in age-matched control (a), STZ-induced diabetic (b) and cholesterol-fed (c) mice. Acetylcholine (10^{-5} M) was applied at the dots.

and LDL cholesterol levels STZ-induced diabetic mice (Table 1). Furthermore, plasma total cholesterol and LDL cholesterol levels were significantly raised in cholesterol-fed mice and the increased cholesterol levels were significantly reduced by chronic administration of cholestyramine (300 mg kg^{-1}, p.o., daily for 10 weeks (Table 2). Such chronic administration of cholestyramine had no effect on the plasma glucose level (Table 1).

Effect of LPC on ACh-induced increase in $[Ca^{2+}]_i$ in the endothelium

LPC did not affect the resting $[Ca^{2+}]_i$ in the endothelium-intact aorta. After exposure to LPC (5 μg ml^{-1}) for 30 min, the ACh-induced increase in $[Ca^{2+}]_i$ was significantly attenuated in control mice (Figure 7). Significant effects of LPC were observed in both Ca^{2+}-free and normal KHS (Figure 5).

Discussion

The main finding in the present study was that the ACh-induced influx of Ca^{2+} into the aortic endothelium was significantly reduced in both STZ-induced diabetic and cholesterol-fed mice and that this weaker influx of Ca^{2+} was restored to normal by the chronic administration of cholestyramine. LPC reduced the influx of Ca^{2+} into the endothelium and Ca^{2+} release from the store sites in aortae from normal mice, suggesting that LPC may affect Ca^{2+} mobilization in the endothelium.

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

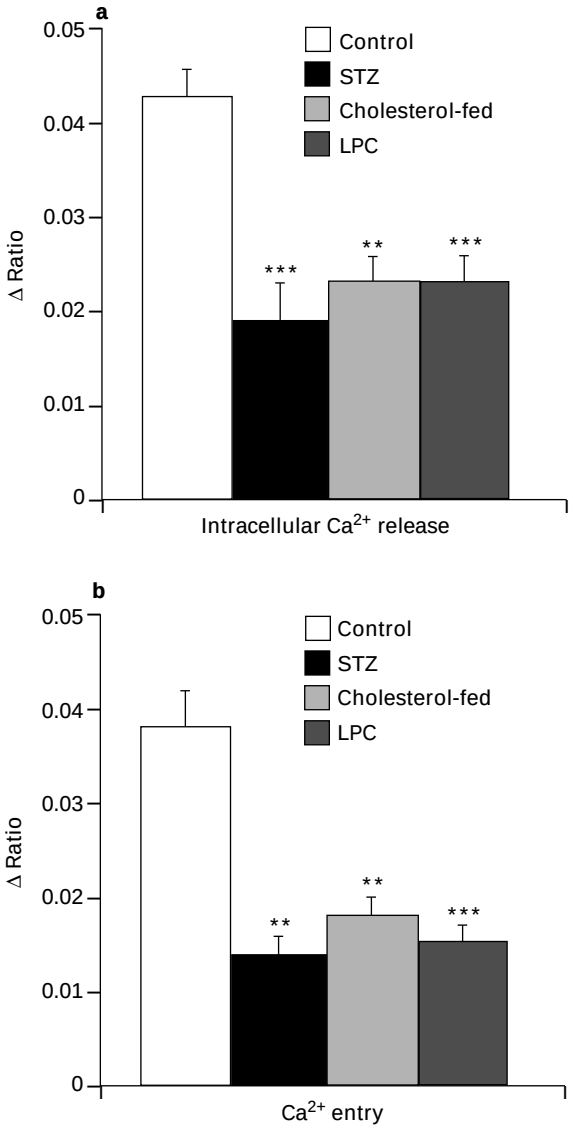

Figure 5 ACh (10^{-5} M)-induced increase in intracellular Ca^{2+} in Ca^{2+}-free medium (a) and normal KHS (b) in age-matched control ($n=8$), STZ-induced diabetic ($n=8$), cholesterol-fed ($n=8$) and control mice treated with lysophosphatidylcholine (LPC) (5 μg ml^{-1}) ($n=8$). Since the ACh-induced increase in intracellular Ca^{2+} was fairly steady at 10 min after its application in normal KHS (as can be seen in Figure 4), intracellular Ca^{2+} in normal KHS was measured at this point. Ca^{2+} entry in Ca^{2+}-free medium was measured at the peak of the response. $^{**}P<0.01$, $^{**}P<0.001$ vs control.

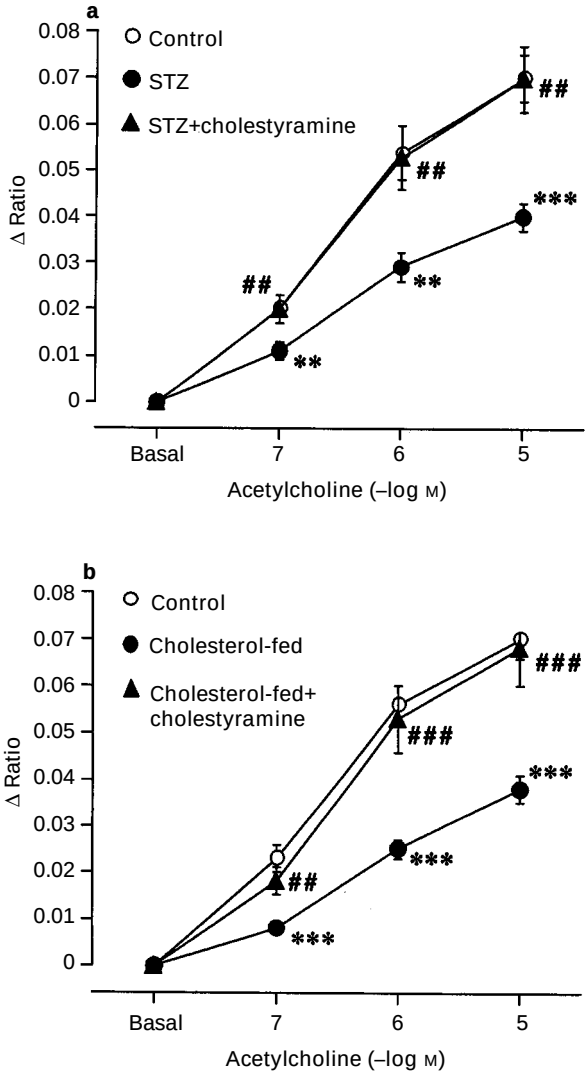

Figure 6 Dose-response curves for ACh-induced increase in intracellular Ca^{2+} in endothelium-intact aortae from STZ-induced diabetic mice given or not given cholestyramine, and from cholesterol-fed mice given or not given cholestyramine. (a) Age-matched control mice ($n=7$); STZ-induced diabetic mice ($n=9$); cholestyramine-treated STZ-induced diabetic mice ($n=8$). (b) Age-matched control mice ($n=7$); cholesterol-fed mice ($n=11$); cholestyramine-treated cholesterol-fed mice ($n=8$). Each data point represents the mean and vertical lines show s.e.; the s.e. is shown only when it exceeds the dimension of the symbol used $^{**}P<0.01$, $^{***}P<0.001$ vs control; $\#\#P<0.01$, $\#\#\#P<0.001$ for STZ group vs cholestyramine-treated STZ group and for cholesterol-fed group vs cholestyramine-treated cholesterol-fed group.

human atherosclerotic coronary arteries (Freiman *et al*., 1986; Habib *et al*., 1986; Verbeuren *et al*., 1986; Bossaller *et al*., 1987; Jayakody *et al*., 1987; Forsterman *et al*., 1988; Shimokawa & Vanhoutte, 1989; Simon *et al*., 1983). Impaired endothelium-dependent relaxation has also been observed in the blood vessels of genetically diabetic rats (Durante *et al*., 1988; Miyata *et al*., 1992; 1993). STZ-induced diabetic rats (Oyama *et al*., 1986; Pieper & Gross, 1988; Kamata *et al*., 1989a,b; 1992; Abiru *et al*., 1993; Poston & Taylor, 1995) and alloxan-induced diabetic rabbits (Tesfamariam *et al*., 1989; Abiru *et al*., 1990a,b; 1991).

Impaired endothelium-dependent relaxation in STZ-induced diabetic and cholesterol-fed mice might be due to (1) decreased influx of Ca^{2+} into the endothelium or decreased release of Ca^{2+} from its storage sites, (2) a decreased content of calmodulin in the endothelium, (3) a decreased content or inactivation of NO synthase, (4), decreased diffusion of NO into the smooth muscle and/or (5) enhanced NO inactivation by superoxide anions. In fact, we have recently found that the mRNA for superoxide dismutase (SOD) is markedly decreased in STZ-induced diabetic rats (Kamata & Kobayashi, 1996), leading to reduced inactivation of superoxide anions by SOD. Thus, NO inactivation may be enhanced by the increase in superoxide anions in the diabetic state. In the present study, we found that there was a reduced influx of Ca^{2+} into the aortic endothelium in both STZ-induced diabetic and cholesterol-fed mice. In endothelium-denuded aortic rings, ACh did not evoke an increase in $[Ca^{2+}]_i$, indicating that ACh-induced increase in $[Ca^{2+}]_i$ occurred only in the endothelium, and not in the smooth muscle. The transient increase in $[Ca^{2+}]_i$ in Ca^{2+}-free medium and the long-lasting increase in $[Ca^{2+}]_i$ in normal KHS were both significantly decreased in STZ-induced diabetic mice and in cholesterol-fed mice. These results strongly suggest that a decrease in Ca^{2+} influx into the endothelium and a decreased release of Ca^{2+} from endoplasmic reticulum (Ca^{2+} store sites) are both involved in the impairment of endothelium-dependent relaxation in these mice.

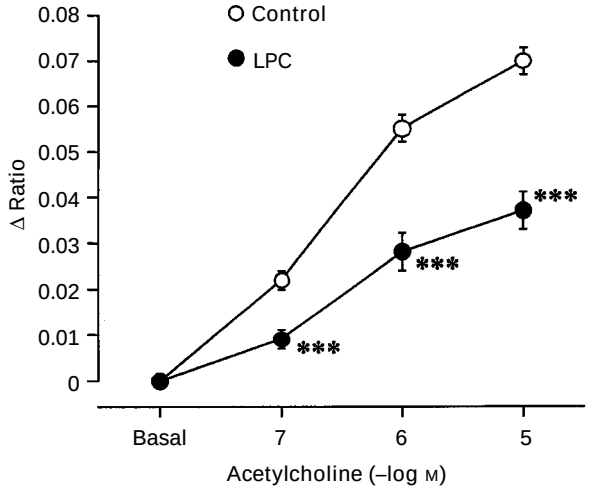

Figure 7 Dose-response curves for ACh-induced increase in intracellular Ca^{2+} in the endothelium of aortae from normal mice. Data were collected in the absence ($n=14$) and presence ($n=10$) of LPC (5 μg ml^{-1}). Each data point represents the mean and vertical lines show s.e.; the s.e. is shown only when it exceeds the dimension of the symbol used. ***$P<0.001$ vs control.

It has been shown that high glucose inhibits cytosolic Ca^{2+} signalling in rat mesangial cells (Mene *et al*., 1993). Furthermore, data obtained from cultured endothelial cells indicate that an elevated glucose level itself results in enhanced store release of Ca^{2+} and greater Ca^{2+} influx (Graier *et al*., 1996), though other studies have only identified an elevation in basal $[Ca^{2+}]_i$ levels (Sobrevia *et al*., 1996) or no change in NO production (and so presumably no change in $[Ca^{2+}]_i$, Mancusi *et al*., 1996). In the present study, we incubated aortic rings with a high concentration of glucose for 6 h, and found that this did not change the ACh-induced increase in $[Ca^{2+}]_i$. The apparent discrepancies between the various results mentioned above might be attributable to differences in the species, in the type of cell, or in experimental conditions. Although our results suggest that high glucose does not impair Ca^{2+} mobilization in the endothelium of the mouse aorta, this requires further investigation.

Cholestyramine is the chooride salt of a basic anion-exchange resin. Cholestyramine binds bile acids in the intestine

Table 1 Plasma levels of total cholesterol, HDL cholesterol, LDL cholesterol and glucose in age-matched controls and STZ-induced diabetic mice

Animals	*Total cholesterol* (mg dl^{-1})	*HDL cholesterol* (mg dl^{-1})	*LDL cholesterol* (mg dl^{-1})	*Glucose* (mg dl^{-1})
Control ($n=7$)	135.4±6.0	88.5±4.0	46.9±2.9	218.2±11.0
STZ ($n=9$)	260.6±20.3**	121.6±8.7***	139.0±23.3***	807.0±23.8***
STZ ($n=8$) + cholestyramine	200.2±16.0#	132.7±6.4	67.5±10.0#	892.5±47.1

Values are mean±s.e. ** $P<0.01$, *** $P<0.001$, STZ-induced diabetic mice vs controls; # $P<0.05$, STZ-induced diabetic group vs STZ-diabetic mice receiving cholestyramine.

Table 2 Plasma levels of total cholesterol, HDL cholesterol and LDL cholesterol in age-matched control and cholesterol-fed mice

Animals	*Total cholesterol* (mg dl^{-1})	*HDL cholesterol* (mg dl^{-1})	*LDL cholesterol* (mg dl^{-1})
Control ($n=7$)	141.7±5.9	95.3±5.1	46.4±3.6
Cholesterol-fed ($n=11$)	272.4±24.2**	60.0±4.4	212.5±25.1***
Cholesterol-fed ($n=7$) + cholestyramine	192.4±7.0#	69.1±10.2	123.3±13.3#

Values are mean±s.e. ** $P<0.01$, *** $P<0.001$, cholesterol-fed groups vs controls; # $P<0.05$, cholesterol-fed mice vs cholesterol-fed mice receiving drug.

and there is consequently a large increase in the faecal excretion of the acids. Cholestyramine also increases the activity of 7-α-hydroxylase, which is the rate-limiting enzyme in bile acid formation (Grundy *et al*., 1971). These findings suggest that cholestyramine, by stimulating 7-α-hydroxylase activity and thus enhancing bile acid synthesis, and also by enhancing LDL-receptor binding, may have the ability to influence the development of atherosclerosis (Shepherd *et al*., 1980; Kovanen *et al*., 1981). We recently showed that in cholesterol-fed and STZ-induced diabetic mice, plasma total cholesterol and LDL cholesterol levels were significantly increased, and that the increased cholesterol levels could be normalized by the chronic administration of cholestyramine (Kamata *et al*., 1996). Moreover, the endothelium-dependent relaxation of aortic rings induced by ACh was significantly attenuated in cholesterol-fed and STZ-induced diabetic mice, and the impaired endothelium-dependent relaxation could be normalized by the chronic administration of cholestyramine. These results suggest (i) that the endothelial dysfunction seen in cholesterol-fed and STZ-induced diabetic mice is due to the increased LDL, (ii) that normal endothelium-dependent relaxation may be preserved by the chronic administration of cholestyramine and (iii) that this effect of cholestyramine may be achieved, at least in part, through a lowering of the serum LDL levels.

Endothelial 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 both in the alteration of various endothelial functions (Kugiyama *et al*., 1990; Rajavashisth *et al*., 1990; Witztum & Steinber, 1991; Flavahan, 1992) and in the initiation of atherosclerosis (Steinberg *et al*., 1984). LPC, which is transferred from oxidized LDL to the endothelial surface membrane, is involved in the mechanism underlying the endothelial functional alterations caused by oxidized LDL (Kugiyama *et al*., 1990; 1992; Flavahan, 1993; Sugiyama *et al*., 1994). Indeed, we earlier confirmed that the endothelium-dependent relaxation of aortic strips induced by ACh was significantly attenuated by pretreatment with LPC (Kamata *et al*., 1995). On this basis, the release of LPC from oxidized LDL would be expected to play an important role in altering endothelial function. If this is the case, the following sequence of events can be predicted in cholesterol-fed and STZ-induced diabetic mice: (i) serum LDL levels are increased in cholesterol-fed and STZ-induced diabetic mice, (ii) the increased LDL is oxidized on the endothelium, (iii) LPC is transferred from the oxidized LDL to the endothelial surface, (iv) this LPC inhibits the endothelium-dependent vasodilatation induced by agonists. This final step would occur via a decreased influx of Ca^{2+} into the endothelium (Inoue *et al*., 1992; Kugiyama *et al*., 1992; Miwa *et al*., 1997) and a decreased release of Ca^{2+} from the endoplasmic reticulum within the endothelium and/or an enhanced inactivation of NO by superoxide anions, thereby resulting in endothelial dysfunction in cholesterol-fed and STZ-induced diabetic mice. Indeed, in the present study, we found that the ACh-induced $[Ca^{2+}]_i$ increase was significantly decreased after exposure to LPC. This inhibitory effect of LPC was also found in Ca^{2+}-free medium, suggesting that LPC may affect the release of Ca^{2+} from the store sites as well as Ca^{2+} influx. Furthermore, in the present study, the chronic administration of cholestyramine significantly lowered serum LDL levels. This cholesterol-lowering effects of cholestyramine may improve the influx of Ca^{2+} into the endothelium or the release of Ca^{2+} from store sites.

An increased urinary excretion of Ca^{2+} (Watkins *et al*., 1985) or no difference in the plasma level of Ca^{2+} (Ismail *et al*., 1990; Hoogwerf *et al*., 1992) has been demonstrated on chronic administration of cholestyramine. Although an improvement in Ca^{2+} mobilization in STZ-induced diabetic and cholesterol-fed mice on chronic administration of cholestyramine might have an effect on Ca^{2+} metabolism, further investigation is required on this point.

In conclusion, we have demonstrated a weaker ACh-induced influx of Ca^{2+} into the aortic endothelium of both STZ-induced diabetic and cholesterol-fed mice and that the decreased influx of Ca^{2+} can be restored to normal by the chronic administration of cholestyramine. LPC reduced the influx of Ca^{2+} into the endothelium and Ca^{2+} release from storage in aortae from normal mice, suggesting that LPC may affect Ca^{2+} mobilization in the endothelium.

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