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Relationship among cholesterol, superoxide anion and endothelium-dependent relaxation in diabetic rats

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Abstract

The purpose of the present study was to investigate the time course of changes in plasma low-density lipoprotein (LDL) cholesterol, tissue lipid peroxidation, the expression of hepatic LDL-receptor mRNA and aortic superoxide dismutase, and the relaxation response to acetylcholine in streptozotocin-induced diabetic rats. Plasma cholesterol and LDL levels were significantly increased in both 4- and 10-week diabetic rats. The tissue malonic dialdehyde content in aortas was increased in 10-week compared to 1- or 4-week diabetic rats. The expression of mRNA for LDL receptor mRNA in the liver showed a decrease in both 4- and 10-week diabetic rats. Hepatic LDL-receptor binding activity decreased significantly in 10-week diabetic rats, and decreased binding activity in diabetic rats was improved by the chronic administration of cholestyramine. The relaxation responses to acetylcholine in helical strips of the aorta precontracted with noradrenaline were significantly decreased in 10-week, but not in 1- or 4-week streptozotocin-induced diabetic rats. The decreased relaxation response to acetylcholine was improved by chronic cholestyramine. Both the expression of Mn-superoxide dismutase mRNA and the activity of superoxide dismutase in the aorta were decreased in 10-week, but not in 4-week diabetic rats. From time-course studies, our data suggest that not only increased LDL cholesterol but also decreased activity of superoxide dismutase are responsible for the decreased relaxation response induced by acetylcholine.

Keywords: Diabetes; LDL (low-density lipoprotein); LDL receptor; Plasma glucose; Lipid peroxidation; Relaxation; Superoxide dismutase; Superoxide anion

1. Introduction

Vascular disease is a complicating feature of diabetes mellitus in humans. An accumulating body of evidence indicates that the relaxation responses of aortic strips to endothelium-dependent agents are weaker in streptozotocin-induced diabetic rats than in non-diabetic rats (Oyama et al., 1986; Pieper and Gross, 1988; Kamata et al., 1989; Poston and Taylor, 1995). Recently, we demonstrated (i) that endothelium-dependent relaxation was significantly attenuated in aortic rings from streptozotocin-induced diabetic mice, and (ii) that the chronic administration of the cholesterol-lowering drug, cholestyramine, both reduced the plasma low-density lipoprotein (LDL) level and normalized the endothelium-dependent relaxation (Kamata et al., 1996). These results strongly suggest that the endothelial dysfunction seen in streptozotocin-induced diabetic mice is due to the increased levels of LDL cholesterol. Although an elevated plasma level of LDL cholesterol could clearly be a major risk factor for endothelial dysfunction, the underlying mechanisms remain unclear.

Oxidized LDL acts more potently than native LDL in markedly inhibiting endothelium-dependent relaxation of the rabbit aorta (Kugiyama et al., 1990) and pig coronary artery (Simon et al., 1990; Tanner et al., 1991). The oxidative modification of LDL cholesterol is thought to be an important step both in the alteration of a variety of endothelial functions (Kugiyama et al., 1990; Rajavashisth et al., 1990) and in the initiation of atherosclerosis (Steinberg et al., 1984). In several in vitro models of LDL oxidation, addition of superoxide dismutase has been shown to be protective (Beckman et al., 1990; Heinecke et al., 1993; Kawamura et al., 1994), suggesting that the superoxide anion (O_2^-) might be involved in the LDL oxidation process. In fact, O_2^- has been proposed as a primary

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cell-derived oxidant with a role in LDL cholesterol oxidation in both endothelial cells (Steinbrecher, 1988) and smooth muscle cells (Heinecke et al., 1986).

Nitric oxide (NO) is rapidly inactivated by O_2^-. Indeed, it has been reported 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). The idea that increased O_2^- levels have an important role in the abnormal endothelial function seen in diabetes is strengthened by the observation that a variety of pharmacological free-radical scavengers, including superoxide dismutase, improve endothelial dysfunction in arteries from diabetic animals (Hattori et al., 1991; Tesfamariam and Cohen, 1992; Langenstroer and Pieper, 1992; Pieper et al., 1992). Recently, we demonstrated that a rapid destruction of NO by O_2^- may occur in streptozotocin-induced diabetic rats, and that this may be due to a decrease in the expression of the mRNA for Mn^{2+}-superoxide dismutase or Cu^{2+}–Zn^{2+}-superoxide dismutase (Kamata and Kobayashi, 1996). However, few studies of endothelial dysfunction in diabetes have directly assessed the relationship between the expression of the mRNA for superoxide dismutase and the oxidative modification of LDL.

Insulin has been reported to increase LDL-receptor expression in vitro in several types of cultured cells and to markedly diminish its suppression by exogenous LDL (Chait et al., 1979; Wade et al., 1988; Krone et al., 1988). Moreover, in humans insulin appears to increase LDL-receptor expression in vivo, since insulin infusion has been found to accelerate the catabolism of LDL (Mazzone et al., 1984). Thus, one mechanism that might underlie the elevation of plasma LDL cholesterol concentration seen in diabetes is a suppression of LDL-receptor activity as a result of insulin deficiency.

To test this hypothesis, we have investigated changes in plasma LDL levels and in the expression of hepatic LDL-receptor mRNA in both short- and long-term diabetes. In the present study, therefore, we have investigated the relationship between changes in plasma glucose, and plasma LDL cholesterol concentration, tissue lipid peroxide content, the expression of hepatic LDL-receptor mRNA and aortic superoxide dismutase, and the relaxation response to acetylcholine in streptozotocin-induced diabetic rats.

2. Materials and methods

2.1. Animal model of diabetes

Male Wistar rats, 8 weeks old and 220–300 g in weight, received a single injection via the tail vein of streptozotocin 60 mg/kg, dissolved in a citrate buffer. Age-matched control rats were injected with the buffer alone. Food and water were given ad libitum. Streptozotocin-induced diabetic rats received saline or cholestyramine (150 mg/kg, p.o., twice daily for 4 weeks). We administered this drug 6 weeks after streptozotocin injection. 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.

2.2. Measurement of plasma cholesterol, LDL and glucose

Plasma total cholesterol and triglycerides were determined using a commercially available enzyme kit (Wako, Osaka, Japan). High-density lipoprotein (HDL) cholesterol was measured following phosphotungstic-$MgCl_2$ precipitation of apolipoprotein B containing very low-density lipoprotein (VLDL) and LDL (Wako, Osaka, Japan). Plasma LDL was derived from the above data using the Friedewald formula: LDL cholesterol = total cholesterol − HDL − triglyceride/5 (Friedewald et al., 1972). The concentration of glucose in the plasma was determined by the toluidine method (Dubowski, 1962).

2.3. Preparation of tissue fractions for measurement of superoxide dismutase activity and lipid peroxidation

Rat aortas were carefully isolated and cleaned of adhering parenchyma and connective tissue. The aorta was homogenized in 10 vols of 50 mM phosphate buffer, 0.1 mM ethylenediaminetetraacetic acid, pH 7.4, at 4°C for 1 min (4×15 s with a 15-s cooling period in between), using a glass-Teflon homogenizer. The homogenate was filtered through cheese cloth and the filtrate was centrifuged at $400 \times g$ for 5 min. The supernatant was used for measurement of superoxide dismutase activity and lipid peroxidation.

2.4. Measurement of lipid peroxidation in the aortae

In this study, lipid peroxidation was assessed by measuring the tissue content of malondialdehyde, one of the end-products of lipid peroxidation (Yagi, 1976). After centrifugation, the supernatant was mixed with 0.67% thiobarbituric acid in glacial acetic acid. After heating at 95°C for 60 min, fluorescent reaction products were assayed with a spectrofluorometer at an excitation wavelength of 515 nm and an emission wavelength of 553 nm. Freshly diluted tetrametoxypropane, which yields malondialdehyde, was used as a standard and results are expressed as nanomoles of malondialdehyde equivalents.

2.5. Measurement of superoxide dismutase activity

Superoxide dismutase activity was assayed with a previously described indirect inhibition assayed, in which xanthine and xanthine oxidase serve as a superoxide generator, and nitroblue tetrazolium is used as a superoxide indicator (Loven et al., 1982; Mantha et al., 1993). The formazan produced was measured spectrophotometrically at 560 nm. The activity was expressed as units/mg protein. One unit inhibits the rate of reduction of cytochrome *c* by 50% in a coupled system with xanthine and xanthine oxidase at pH 7.8 at 25°C in a 3.0 ml reaction volume.

2.6. Measurement of isometric force

Rats were anesthetized with diethyl ether and killed by decapitation, 1, 4 or 10 weeks after treatment with streptozotocin or buffer. A section of the thoracic aorta from between the aortic arch and the diaphragm was then removed and placed in oxygenated, modified Krebs–Henseleit solution. The solution consisted of (mM): NaCl 118.0, KCl 4.7, $NaHCO_3$ 25.0, $CaCl_2$ 1.8, NaH_2PO_4, $MgSO_4$ 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 bath containing of 10 ml of well-oxygenated (95% O_2, 5% CO_2) KHS at 37°C. One end of the tissue was 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 (determined to be optimum in preliminary experiments). During this period, the Krebs–Henseleit solution in the tissue bath was replaced every 20 min. After equilibration, each aortic strip was contracted with 10^{-7} M noradrenaline. The presence of functional endothelial cells was confirmed by demonstrating relaxations in response to 10^{-5} M acetylcholine. Aortic strips in which 75% relaxation occurred being regarded as tissues with endothelium. The relaxation response to acetylcholine is expressed as a percentage of the contractile force induced by 10^{-7} M noradrenaline. The maximal noradrenaline-induced contraction of aortic strips is enhanced in diabetic rats (Kamata et al., 1988). Hence, for the relaxation studies the aortic strips were precontracted with an equieffective concentration of noradrenaline (5×10^{-8}–3×10^{-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 $5 \times 10^{-8} \approx 3 \times 10^{-7}$ M noradrenaline so that each strip developed a tension of approximately 95 mg/mg tissue in both age-matched controls and diabetic rats. When the noradrenaline-induced contraction reached a plateau level, acetylcholine (10^{-9}–10^{-5} M) was added in a cumulative manner.

2.7. Measurement of the expression of superoxide dismutase mRNA and LDL-receptor mRNA

2.7.1. Oligonucleotides

The following oligonucleotides were used as primers for the reverse transcription polymerase chain reaction (RT-PCR). The respective EMBL data library accession number and position of the PCR product in the coding sequence are given in brackets: rat GAPDH (X02231, position 492–799) oligonucleotide 1; 5′-TCCCTCAA-GATTGTCAGCAA-3′, oligonucleotide 2; 5′AGATC-CACAACGGATACA TT-3′: rat LDL-receptor (X13722, position 5–623) oligonucleotide 3; 5′-CCCAGTGCG-GCGTAGGATTG-3′, oligonucleotide 4; 5′-GAGTGG-CAGCGGAAGRGAGC-3′; rat Mn superoxide dismutase (Y00497, position 329–945) oligonucleotide 5: 5′-CC-CTAAGGGTGGTGGAGAAC-3′, oligonucleotide 6; 5′-GGCCTTATGATGACAGTGAC-3′.

2.7.2. RNA isolation and RT-PCR

RNA was isolated according to the guanidinium method (Chomczynski and Sacchi, 1987). Rat aortas were carefully isolated and cleaned of adhering parenchyma and connective tissue. The tissue (livers or aortas) was homogenized in RNA buffer using a glass-Teflon homogenizer. RNA was quantified by ultraviolet absorbance spectrophotometry. For the RT-PCR analysis, first-strand cDNA was synthesized from total RNA using Oligo $(dT)_{12-18}$ and cDNA Synthesis Kit (Life Sciences). RNA (1 μg was reversely transcribed in a final volume of 20 μl using 12.5 unit AMV reverse transcriptase in the first-strand reaction mix, 12.5 mM dithiothreitol, 0.05 μg Oligo $(dT)_{12-18}$, and 12.5 units RNasin™ RNase inhibitor for 1 h at 42°C, for 7 min at 99°C. Twenty-five PCR cycles (LDL) receptor; 94°C for 1 min, 62°C for 1 min, 72°C for 1 min, Mn superoxide dismutase; 94°C for 1 min, 55°C for 1 min, 72°C for 1 min) were performed in a final volume of 50 μl with half of the reverse transcription 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.

2.7.3. Competitive PCR

The amount of mRNA was measured with competitive PCR techniques using a heterogenous DNA fragment (PCR mimic) as an internal standard (Abe et al., 1995). The PCR mimic was created essentially according to the instructions of the kit (PCR Mimic Construction Kit, Clontech, CA, USA). A part of the PCR mimic (400 bp) was amplified with protruding 20 bp in ends specific for LDL receptor or Mn^{2+}-superoxide primers, respectively. Two-fold dilutions of the PCR mimic (LDL receptor; 4, 2, 1, 0.5, 0.25 amol/μl, Mn^{2+}-superoxide dismutase; 10, 5, 2.5, 1.25, 0.625,

0.3125 amol/μl) were added to the PCR amplification reaction mixture containing a constant amount of the rat cDNA sample. Ten percent of the RT mixture with 4 μg original RNA, obtained from control or diabetic rats, was amplified by PCR in 25 cycles with serial dilution of the PCR mimic. The target cDNA gene and PCR mimic were amplified with the target gene specific primers for LDL receptor or Mn^{2+}-superoxide. Thus, the mimic and target templates compete for the same primers in the same reaction. A semiquantitative evaluation of mRNA levels was made by comparing the amount of each product (target cDNA gene and PCR mimic) after electrophoresis.

2.8. Measurement of LDL receptor activity by ligand blot assay

The liver was homogenized in 4 vols of a buffer (10 mM Tris–HCl, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM PMSF, pH 7.4 buffer). The homogenate was filtered through cheese cloth and the filtrate was centrifuged at $500 \times g$ for 10 min at 4°C. The supernatant was sonicated at 4°C and the proteins were solubilized in 30 mM CHAPS. The membrane preparation was mixed with loading buffer (10% glycerol, 0.5% SDS, 2 mM $CaCl_2$, 0.05% bromophenol blue and 50 mM Tris–HCl, pH 6.8) and the proteins (10 μg protein/lane) were separated by SDS-PAGE (5.0% polyacrylamide gels). The separated proteins were electrophoretically transferred to PVDF membranes. After a 1-h preincubation in blocking buffer (Block Ace, Snow Brand, Japan), the filters were incubated for 4 h with biotin-labeled rabbit β-migrating very low-density lipoprotein (b-VLDL, 10 μg/ml) in the absence or presence of non-labeled LDL (200 μg/ml) and developed with biotinylated streptavidin peroxidase complexes. Bands were visualized with 3′,3′-diaminobenzidine tetrahydrochloride substrate. The amount of LDL receptor and VLDL receptor bands, 120–130 kDa, were quantitated by scanning densitometry of dried membranes. For quantitation of hepatic LDL receptor, this ligand blot assay uses well-labeled β-VLDL (Wade et al., 1988; Rudling and Angelin, 1993a,b). We also used β-VLDL as the ligand; however this ligand is known to bind to the VLDL receptor as well as the LDL receptor (Takahashi et al., 1995). This new receptor, designated VLDL receptor, was shown to be highly similar to the LDL receptor and to bind with VLDL and β-VLDL but not to bind LDL (Takahashi et al., 1992). Thus, LDL receptor activity was derived from the above data: LDL receptor activity = expression band (arbitrary unit) with b-VLDL as ligand − expression band with b VLDL presence of non-labeled LDL.

2.9. Drugs

Streptozotocin and (−)-noradrenaline hydrochloride were purchased from Sigma (St. Louis, MO, USA). Acetylcholine chloride was purchased from Daiichi Pharmaceuticals (Tokyo, Japan). All drugs were dissolved in saline, except where otherwise noted. All concentrations are expressed as the final molar concentration of the base in the organ bath.

2.10. Statistical analysis

Data are expressed as the means ± S.E.M. The statistical significance of differences was assessed using Dunnett's test for multiple comparison, after a one-way analysis of variance. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Plasma glucose in age-matched controls and in 1-, 4- and 10-week streptozotocin-induced diabetic rats

In rats 1, 4 and 10 weeks after treatment with streptozotocin, the concentration of glucose in the plasma was

Table 1
Changes in various parameters in controls, streptozotocin-induced diabetic rats and diabetic rats pretreated with cholestyramine

	1-week Cont	1-week DM	4-week Cont	4-week DM	10-week Cont	10-week DM	10-week DM cholestyramine
Body weight (g)	276 ± 2(6)	270 ± 6(6)	369 ± 5(8)	258 ± 6(12)c	489 ± 13(15)	275 ± 16(18)c	263 ± 12(9)
Plasma glucose (mg/dl)	158 ± 7(6)	426 ± 28(8)c	150 ± 6(6)	602 ± 27(8)c	138 ± 3(8)	594 ± 50(12)c	557 ± 42(8)
Ketone bodies (mg/dl)	N.T.	N.T.	N.T.	N.T.	1.0 ± 0.3(6)	24.7 ± 4.1(6)c	N.T.
Triglyceride (mg/dl)	155 ± 16(6)	160 ± 23(8)	124 ± 14(6)	271 ± 42(8)a	157 ± 15(6)	277 ± 56(12)a	382 ± 36(8)
Total cholesterol (mg/dl)	112 ± 7(6)	120 ± 6(8)	105 ± 5(6)	194 ± 23(8)b	119 ± 4(6)	205 ± 22(12)c	170 ± 9(8)
LDL cholesterol (mg/dl)	29 ± 2(6)	26 ± 4(8)	23 ± 4(6)	67 ± 17(8)a	19 ± 3(6)	62 ± 15(12)b	33 ± 4(8)d
Tissue MDA (nmol/mg protein)	N.T.	0.62 ± 0.08(6)	N.T.	0.69 ± 0.09(6)	N.T.	1.33 ± 0.09(6)c	N.T.

Cont, control rats; DM (diabetes mellitus), streptozotocin-induced diabetic rats; 10-week DM cholestyramine, streptozotocin-induced diabetic rats that had been treated with cholestyramine (150 mg/kg, p.o. twice daily for 4 weeks). MDA, malondialdehide.
N.T. = not tested. Number of determinations is shown in within parentheses.
(a) $P < 0.05$, (b) $P < 0.01$, (c) $P < 0.001$ vs. Cont, (d) $P < 0.05$ vs. DM.

elevated significantly. Various parameters are summarized in Table 1.

3.2. Plasma total cholesterol and LDL cholesterol concentrations in age matched controls and in 1-, 4- and 10-week streptozotocin-induced diabetic

The concentration of total cholesterol in the plasma was unchanged in 1-week diabetic rats, but 4 or 10 weeks after treatment with streptozotocin it was elevated significantly (Table 1). The concentration of LDL cholesterol was unchanged in 1-week diabetic rats, but 4 or 10 weeks after treatment with streptozotocin it was elevated significantly (Table 1). Chronic administration of cholestyramine (150 mg/kg) lowered LDL cholesterol concentrations as shown in Table 1.

3.3. Tissue malondialdehyde content in 1-, 4- and 10-week streptozotocin induced diabetic rats

The malondialdehyde content was found to be markedly increased in the aortas from 10-week diabetic rats, whereas it was not changed in aortas from 1- and 4-week diabetic rats (Table 1).

3.4. Expression of hepatic LDL-receptor mRNA

Using RT-PCR on the total RNA isolated from the liver of 1-, 4- and 10 week diabetic rats and age-matched controls, we examined the changes occurring in the expression of mRNA for the LDL receptor. We observed that the expression was markedly decreased in the livers of 4- and 10-week diabetic rats, whereas it was not changed in livers from 1-week diabetic rats (Fig. 1A). A semiquantitative evaluation of mRNA levels was carried out by comparing each product after electrophoresis. By this method, we found that the LDL-receptor mRNA level in the liver was significantly decreased in both the 4- and 10-week diabetic rats (Fig. 1B).

3.5. Activity of hepatic LDL-receptor by ligand blotting

Ligand blots were performed with hepatic membrane extracts from 10 week control, 10-week diabetic rats and diabetic rats pretreated with cholestyramine. The mobility of the LDL-receptor and VLDL-receptor proteins corresponded to a molecular mass of 120–130 kDa. Hepatic LDL receptor bindings assessed by ligand blots was significantly decreased in 10-week diabetic rats. This decreased binding in diabetic rats was improved by the chronic administration of cholestyramine (150 mg/kg, p.o. twice daily for 4 weeks) as shown in Fig. 1C.

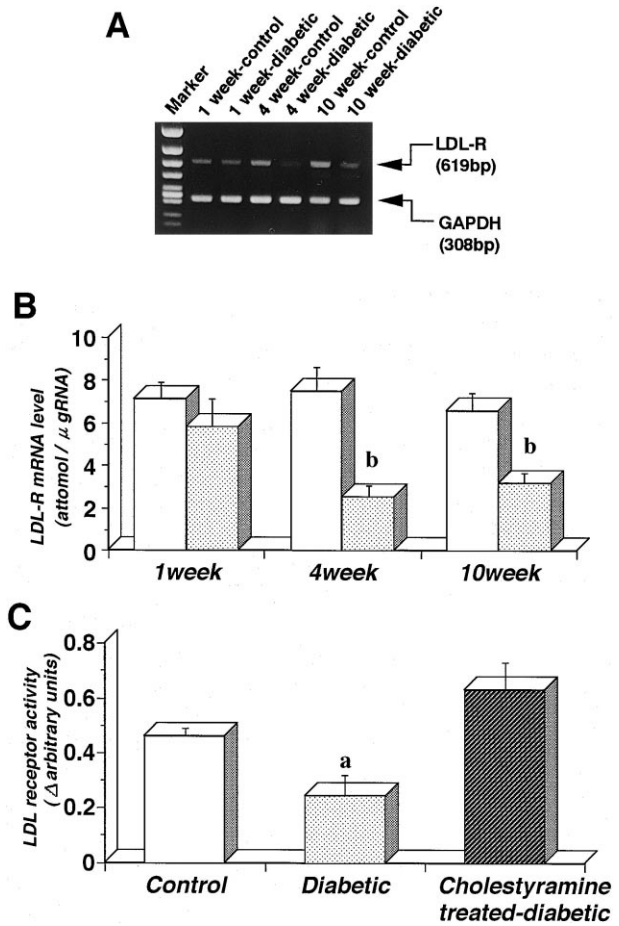

Fig. 1. RT-PCR (A) competitive PCR (B) and ligand blot (C) assay of expression of hepatic LDL receptor in age-matched controls and in 1-, 4- and 10-week diabetic rats and diabetic rats treated with cholestyramine. (A) Upper lane, LDL receptor mRNA expression (619 bp); lower lane, GAPDH mRNA expression (308 bp); left lane, ϕX174/*Hinc*II digest molecular size marker. (B) Control rats ($n = 5$, open column); streptozotocin-induced diabetic rats ($n = 5$, dot column). (C) 10-week control rats ($n = 8$, open column); 10-week streptozotocin-induced diabetic rats ($n = 8$, dot column); Diabetic rats pretreated with cholestyramine (150 mg/kg, p.o. twice daily for 4 weeks) ($n = 6$, hatched column). Values are means $\pm$ S.E. of five to eight determinations (amol/μg RNA or Δ arbitrary units.). Statistically different from age-matched control, $^{a}P < 0.05$, $^{b}P < 0.01$.

3.6. Relaxation in response to acetylcholine in age-matched controls and in 1-, 4- and 10-week streptozotocin-induced diabetic rats

When the noradrenaline (5×10^{-8}–3×10^{-7} M) induced contraction had reached a plateau, acetylcholine (10^{-9}–10^{-5} M) caused a concentration dependent relaxation. In 1- or 4-week diabetic rats, the acetylcholine induced relaxation of the aortic strips was not different from that observed in age-matched controls (Fig. 2A). By contrast, the relaxations caused by acetylcholine were significantly decreased in aortic strips from 10-week diabetic rats (Fig. 2B). The decreased response to acetylcholine

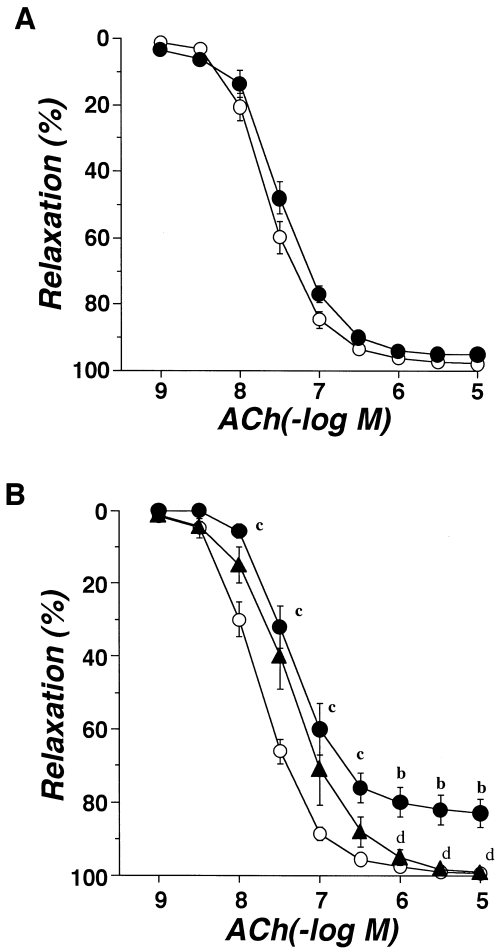

Fig. 2. Concentration–response curves for acetylcholine-induced relaxation of aortic strips obtained from age-matched control rats and streptozotocin induced diabetic rats. (A) Age-matched controls ($n=6$, ○); 4-week streptozotocin-induced diabetic rats (n = 6, ●). (B) Age-matched controls ($n=6$, ○); 10-week streptozotocin-induced diabetic rats ($n=6$, ●); 10-week streptozotocin-induced diabetic, cholestyramine-treated rats ($n=6$, ▲). The ordinate shows the relaxation of aortic strips as a percentage of the contraction induced by an equieffective concentration of noradrenaline (5×10^{-8}–3×10^{-7} M). Each data point on the graph represents the mean ± S.E. of six experiments; the S.E. is included only when it exceeds the dimension of the symbol used. Statistical difference from age-matched control: $^{b}P<0.01$, $^{c}P<0.001$, controls vs. streptozotocin-induced diabetic rats; $^{d}P<0.01$, streptozotocin-induced diabetic rats vs streptozotocin-induced diabetic, cholestyramine-treated rats.

was improved by the chronic administration of cholestyramine (150 mg/kg, p.o. twice daily for 4 weeks) as shown in Fig. 2B.

3.7. Expression of Mn^{2+}-superoxide dismutase mRNA

When the changes occurring in the expression of Mn superoxide dismutase mRNA in the aorta were investigated, Mn superoxide dismutase mRNA expression was found to be markedly decreased in aortas from 10-week diabetic rats, whereas it was not changed in aortas from 1- and 4-week diabetic rats (Fig. 3A). A semiquantitative evaluation of mRNA levels was carried out by comparing each product after electrophoresis. By this method, we found that the Mn^{2+}-superoxide dismutase mRNA was significantly decreased in the aortas of 10-week diabetic rats (Fig. 3B).

3.8. Activity of superoxide dismutase

The activity of superoxide dismutase in the aorta was found to be markedly decreased in the 10-week diabetic rats, whereas it was not changed in 4-week diabetic rats (Fig. 3C).

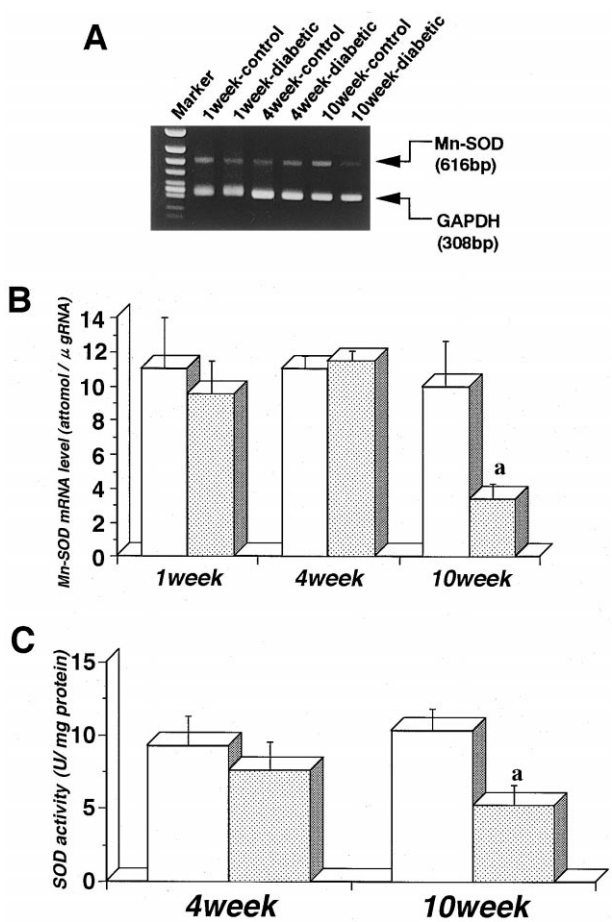

Fig. 3. RT-PCR (A), competitive PCR (B) assay of expression of aortic Mn^{2+}-superoxide dismutase mRNA and superoxide dismutase activity in age matched control, and in 1-, 4-, 10-week diabetic rats. (A) Upper lane, Mn^{2+}-superoxide dismutase mRNA expression (616 bp); lower lane, GAPDH mRNA expression (300 bp); left lane, ϕX174/*Hin*cII digest molecular size marker. (B) Control rats ($n=5$, open column); streptozotocin-induced diabetic rats ($n=5$, dot column). (C) Control rats ($n=5$, open column); streptozotocin-induced diabetic rats ($n=5$, dot column). Values are means ± S.E. of five determinations (amol/μg RNA). Statistically different from age-matched control, $^{a}P<0.05$.

4. Discussion

The main conclusion from the present study is that oxidative modification of LDL cholesterol may be related to endothelial dysfunction in the aorta of the streptozotocin-induced diabetic rat, and that this may be due to a decrease in the tissue content of superoxide dismutase. In addition, our results suggest that the increased plasma LDL levels seen in long-term diabetic rats may cause down-regulation of LDL receptors in the liver. We also found that acetylcholine-induced endothelium-dependent relaxation was significantly attenuated in 10-week, but not in 1- or 4-week diabetic rats. The decreased relaxation response to acetylcholine and LDL receptor activity was improved by the chronic administration of cholestyramine.

Recently, we demonstrated that the impaired endothelium-dependent relaxation seen in streptozotocin-induced diabetic mice was due to an increase in LDL cholesterol (Kamata et al., 1996). In the present study, plasma LDL levels were significantly increased in both 4- and 10-week streptozotocin-induced diabetic rats. These results support the idea that the endothelial dysfunction observed here in 10-week diabetic rats may be due to the increased plasma LDL level. However, the plasma LDL level was significantly increased in 4-week diabetic rats, whereas the acetylcholine induced relaxation was unchanged. This result may suggest that there is not a direct relationship between the plasma LDL level itself and the extent of endothelial dysfunction. In this study, the tissue malondialdehyde content in aortas was increased in 10-week compared to 1- or 4-week diabetic rats. When plasma LDL concentrations are high, LDL gradually enters and accumulates in the arterial intima. As mentioned in the Introduction, there is reason to believe that superoxide generated by arterial smooth muscle and endothelium may contribute to the oxidation of LDL cholesterol and play a central role in the modification of LDL and the consequent development of endothelial dysfunction. These results suggest that the endothelial dysfunction in diabetes may be due to the accumulation of oxidized LDL cholesterol in the arterial intima.

Mn^{2+}-superoxide dismutase, localized in the mitochondrial matrix and partially released into the serum, has the essential function of reducing the concentration of superoxide radicals both during normal oxidative metabolism and during periods of oxidative stress. In the present study, in which we used competitive PCR, the expression of Mn^{2+}-superoxide dismutase mRNA was found to be significantly decreased in the aortas of 10-week diabetic rats. 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. Indeed, atherosclerotic rabbits treated with polyethylene-glycolated superoxide dismutase or liposome-encapsulated superoxide dismutase have increased vascular superoxide dismutase activity and improved endothelium-dependent relaxation in response to acetylcholine (Mugge et al., 1991; White et al., 1994). Similarly, treatment of animals with antioxidant, vitamin C, tocopherol or probucol prevents the endothelial dysfunction produced by hypercholesterolemia and streptozotocin-induced diabetes (Mantha et al., 1993; Simon et al., 1993; Keegan et al., 1995; Keaney et al., 1995; Ting et al., 1996). These studies suggest that antioxidants prevent the development of endothelial dysfunction by inhibiting the oxidative modification of LDL.

Vascular superoxide dismutase activity exerts considerable influence over the local activity of endothelium-derived relaxing factor (EDRF)- and NO-dependent vasodilators. Isolated arteries exposed to diethyldithiocarbamate, a potent inhibitor of superoxide dismutase, fail to relax normally to the EDRF releasing agent, acetylcholine, or NO-dependent vasodilators such as nitroprusside or nitroglycerin (Omar et al., 1991; Pagano et al., 1993). Several studies have suggested that the impaired endothelium-dependent relaxation seen in diabetes may stem from a greater production of superoxide anions (Hattori et al., 1991; Langenstroer and Pieper, 1992; Pieper et al., 1992). Thus, an imbalance in the activity of superoxide dismutase and the production of superoxide anions may predispose to impaired endothelium-dependent relaxation and altered vascular reactivity. Recently, we suggested that a rapid destruction of NO by superoxide anions may occur in the streptozotocin-induced diabetic rat, and that this may be due to a decrease in the expression of the mRNA for Mn^{2+}-superoxide dismutase or Cu^{2+}–Zn^{2+}-superoxide dismutase (Kamata and Kobayashi, 1996). In the present study, the acetylcholine-induced endothelium-dependent relaxation was significantly attenuated in 10-week diabetic rats, and the expression of Mn^{2+}-superoxide dismutase mRNA and activity of superoxide dismutase was also significantly decreased at the same time. These results strongly suggest that endothelial dysfunction is caused by a decrease in the activity of superoxide dismutase in the aorta of streptozotocin-diabetic rats.

It has been shown that the level of plasma LDL is directly linked to the extent of LDL-receptor-mediated uptake in the liver (Carew et al., 1982; Koelz et al., 1982), and that experimental alteration of hepatic LDL receptor activity can result in a corresponding alteration of plasma LDL cholesterol concentration (Kovanen et al., 1981). In the present study, we found that 1-week diabetic rats showed no change in LDL-receptor expression. In line with these findings, other studies have demonstrated that insulin deficiency in short-term diabetic rats had no effect on hepatic LDL-receptor expression (Nakai et al., 1985; Swami et al., 1996). The concentration of LDL cholesterol in the plasma did not change in 1-week diabetic rats, although it was significantly increased in both 4- and

10-week diabetic rats. When the changes occurring in the expression of LDL-receptor mRNA were determined, rather than plasma LDL concentrations, the expression in the liver was found to be decreased in both 4- and 10-week diabetic rats. Furthermore, we found that hepatic LDL-receptor binding activity was significantly decreased in 10-week diabetic rats. This decreased binding activity was improved by the chronic administration of cholestyramine. It has been reported that the increased LDL cholesterol levels in diabetic mice are restored to normal by the chronic administration of cholestyramine (Kamata et al., 1996). These results suggest that long-term insulin deficiency and hyperglycemia in the streptozotocin-induced diabetic rat are associated with an increase in plasma LDL cholesterol, and that this may be due to a reduction in the expression of the LDL receptor in the liver. It has been reported that insulin exerts a stimulatory effect on LDL receptor mRNA (Chait et al., 1979; Wade et al., 1988). Troglitazone, which is a new ant diabetic agent and enhances insulin action at the receptor in peripheral tissues and in liver (Fujiwara et al., 1988; Ciaraldi et al., 1990), has been reported to enhance LDL binding by increasing LDL receptor mRNA levels (Rayyes and Florén, 1998). An alternative explanation for the increase in plasma LDL cholesterol concentration is that insulin deficiency in streptozotocin-induced diabetic rats may lead to a reduction in the number of LDL receptors in the liver, thereby resulting in an increase in plasma LDL cholesterol concentration. However, it should be noted that, in addition to the lower insulin levels, several other hormonal changes occur in vivo streptozotocin-induced diabetes. In fact, the results of several recent studies have suggested alternative mechanisms for sterol-independent control of hepatic LDL-receptor activity (Mazzone et al., 1984; Rudling and Angelin, 1993a,b). Signalling by specific serum hormones may be able to override sterol dependent transcriptional repression in liver-derived cells. In addition, levels of counter-regulatory hormones, such as catecholamines, adrenocorticotropic hormone (ACTH), glucagon, etc., are markedly higher in the diabetic condition. Thus, it is possible that the reduction seen in hepatic LDL receptor expression may be due to changes in the plasma level of these hormones. It is unclear at present, however, which factors might be responsible for decreasing the level of LDL receptor mRNA in the liver. This effect might result from changes in the insulin level, or in the level of any of several other hormones.

To conclude, one possible explanation that is consistent with the observed changes is follows. Streptozotocin increases plasma glucose levels. The low concentration of insulin and the high plasma glucose concentration then increase the concentration of plasma LDL cholesterol, and the increased LDL concentration may cause down-regulation of the expression of LDL. These conclusions are supported by the finding that chronic administration of cholestyramine lowered LDL receptor activity. Long-term, a high plasma glucose concentration also reduces the expression of Mn superoxide dismutase mRNA and the activity of superoxide dismutase in the aorta. The decreased superoxide dismutase may inactivate NO and oxidize LDL, leading to the accumulation of oxidized LDL which may impair the activity of the endothelium, thereby resulting in the observed endothelial dysfunction. These conclusions are supported by time-course measurement of LDL cholesterol concentrations and its receptor levels, superoxide anion levels and the endothelium-dependent relaxation in streptozotocin-induced diabetic rats. Alternatively, the changes in aortic Mn^{2+}-superoxide dismutase mRNA levels or superoxide dismutase activity are primary defects rather than secondary to some phenomenon such as overwhelming oxidative stress. Further studies are required on these points.

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