Endothelial dysfunction in streptozotocin-diabetic rats is not reversed by dietary probucol or simvastatin supplementation

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Summary Oxidative stress and dyslipidaemia are key features of diabetes mellitus and may be involved in mediating the vascular endothelial dysfunction associated with this disease. The aim of this study was to examine the effect of dietary lipid-lowering and antioxidant agents on vascular endothelial function and oxidative stress. Diabetic male Sprague-Dawley rats (i.v. streptozotocin, 45 mg/kg) were fed for 4 weeks on a standard diet or on a diet supplemented with either the lipid-lowering antioxidant probucol (1 % w/w in diet) or the 3-hydroxy 3-methylglutaryl coenzyme-A (HMG-CoA) reductase inhibitor simvastatin (0.01 % w/w in diet). Responses to noradrenaline, acetylcholine, and sodium nitroprusside were assessed in small mesenteric arteries (mean internal diameter $300 \pm 5 \mu\text{m}$, $n = 80$) mounted on a small vessel myograph. Plasma concentrations of total cholesterol and triglycerides were significantly raised in standard-fed diabetic rats and significantly reduced in probucol and simvastatin-fed diabetic rats. 8-epi-

prostaglandin (PG)F_{2α}, an indicator of oxidative stress, was raised in liver and aorta from diabetic rats compared to controls. Probuscol supplementation reduced 8-epi-PGF_{2α} in aorta and liver of diabetic rats but increased 8-epi-PGF_{2α} content in plasma and aorta from control animals. The abnormal relaxation to acetylcholine in arteries from the diabetic rats (pEC_{50} diabetic 6.763 ± 0.172 vs control 7.541 ± 0.175 ; $p < 0.05$) was not improved by probucol or simvastatin. These data, therefore, do not support a role for oxidative stress or dyslipidaemia in mediating the impaired ACh-induced endothelium-dependent relaxation of small mesenteric arteries from the streptozotocin-diabetic rat. [Diabetologia (1998) 41: 157–164]

Keywords Diabetes mellitus, vascular endothelium, lipids, antioxidants, resistance artery, isoprostanes, nitric oxide, acetylcholine, probucol, simvastatin.

Insulin-dependent diabetes mellitus (IDDM) is associated with an increased risk of hypertension, atherosclerosis and disorders of the microcirculation and

there is increasing evidence to suggest that vascular endothelial dysfunction may play a major role. Impaired endothelium-dependent vasodilatation to acetylcholine (ACh) has been reported in aorta [1], small mesenteric [2] and femoral [3] arteries of the streptozotocin (STZ)-rat, and in diabetic patients [4, 5], and is generally associated with reduced availability of nitric oxide (NO). The factors responsible for altered NO synthesis or release are unknown and, although hyperglycaemia undoubtedly contributes [6], there is increasing evidence that oxidative stress coupled with dyslipidaemia [7, 8] could also be important determinants of endothelial dysfunction. Free radicals (which may be generated as a consequence of glucose-induced activation of cyclooxygenase, au-

Received: 4 August 1997 and in revised form: 29 September 1997

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Abbreviations: ACh, Acetylcholine; NA, noradrenaline; SNP, sodium nitroprusside; NO, nitric oxide; PSS, physiological salt solution; NEFA, non-esterified fatty acids; PGF_{2α}, prostaglandin F_{2α}; STZ, streptozotocin; HMG-CoA, 3-hydroxy 3-methylglutaryl coenzyme-A.

tooxidation of glucose or from alterations in transition metal metabolism) may cause structural alteration of DNA and modification of proteins within the endothelial cell [8]. Further damage may arise from free radical induced generation of lipid peroxides, particularly oxidised LDL, which has also been implicated directly in reduced endothelial NO synthesis [9].

Evidence for enhanced oxidative stress in diabetes includes increased concentrations of plasma lipid peroxides, oxidative modification of lipoproteins [10], altered concentrations of endogenous antioxidants [7] and, more recently, from measurements of a stable metabolite of lipid peroxidation, 8-epi-prostaglandin (PG) $F_2\alpha$ [11]. Plasma concentrations of this isoprostane are raised in non-insulin-dependent diabetic (NIDDM) patients [12] and STZ-diabetic rats, and as we have reported recently, are decreased in diabetic rats by antioxidant supplementation [13].

A pivotal role for dyslipidaemia in diabetic endothelial dysfunction has been suggested by a study in STZ-diabetic mice in which administration of the total and LDL-cholesterol lowering drugs taurine [14] and cholestyramine [15] improved endothelium-dependent relaxation in conduit vessels. Other investigations have focused on the potential benefits of the antioxidant and antiatherogenic drug, probucol, and of the 3-hydroxy 3-methylglutaryl (HMG) coenzyme-A reductase inhibitor, simvastatin, in improving either the lipid profile of STZ-diabetic rats [16–18] or vascular dysfunction of hypercholesterolaemic animals [19, 20] and man [21, 22]. Although the many reports of vascular dysfunction in small arteries of diabetic animals and man suggest that reduced endothelium-dependent relaxation may play an important role in the microangiopathies, there has been no study to determine the effect of either probucol or simvastatin on small artery endothelial dysfunction in diabetes.

The aim of this study therefore, was to investigate the effect of probucol and simvastatin dietary supplementation on small mesenteric artery function of the STZ-diabetic rat. In order to evaluate the role of oxidative stress and dyslipidaemia, plasma lipid analyses were performed, and lipid peroxidation assessed by measurement of 8-epi-PGF $_2\alpha$.

Materials and methods

Induction of experimental diabetes. Diabetes was induced in male Sprague-Dawley rats (250–300 g; Bantin and Kingman, Universal Ltd, Aldbrough, Hull, UK) by caudal intravenous injection of STZ (45 mg/kg) dissolved in citrate buffer (0.1 mol/l trisodium citrate, 0.1 mol/l citric acid). Control animals were injected with the vehicle alone. Development of diabetes was confirmed by the presence of glycosuria 3 days after injection. Animals were housed individually and provided with food and water *ad libitum*.

Dietary protocol. Three days after injection of either STZ or vehicle, rats were divided into the following groups: 1). Diabetic rats ($n = 8$), 0.01 % (w/w) simvastatin in standard diet; 2). Diabetic rats ($n = 13$), 1 % (w/w) probucol in standard diet; 3). Diabetic rats ($n = 10$), standard diet; 4). Control rats ($n = 11$), standard diet.

Each diet was administered for 4 weeks. An additional group of control rats was fed the probucol-supplemented diet (control rats, $n = 11$; 1 % probucol (w/w) in standard diet) as, in a previous study of dietary antioxidant supplementation with vitamin E and vitamin C, we demonstrated adverse effects on endothelial function in control rats [23]. In preparation of the diets, standard chow ("rat maintenance diet"; Special Diet Services, Witham, Essex, UK) was ground to a powder and mixed with either simvastatin (ground from tablet form) or probucol powder. The weight of food consumed and animal weight were recorded every 2 days throughout the experimental period.

Plasma and tissue collection. In the fifth week after injection, animals were killed by inhalation of CO $_2$ and cervical dislocation. Samples of liver and sections of the thoracic aorta were removed from control standard-fed rats ($n = 11$), control probucol-fed rats ($n = 11$), diabetic standard-fed rats ($n = 10$) and diabetic probucol-fed rats ($n = 13$) and were immediately snap frozen in liquid nitrogen and stored at -70°C . Blood samples were also obtained from rats in all groups by cardiac puncture and the plasma (for measurement of glucose concentration) stored at -70°C . Additional blood samples were collected into ethylenediamine-tetra-acetic acid (EDTA; 28 mmol/l) for the subsequent measurement of lipids and probucol, or into 3.8 % sodium citrate (blood/anticoagulant ratio 9:1) with 14 $\mu\text{mol/l}$ indomethacin (in 5 % sodium bicarbonate) for assessment of 8-epi-PGF $_2\alpha$. Plasma for lipid and probucol analysis was stored in EDTA (50 mmol/l, -70°C) while blood samples for 8-epi-PGF $_2\alpha$ analysis were centrifuged after standing at 4°C for 45 min (2400 g, 15 min, 4°C) and aliquots of the plasma supernatant stored in butylated hydroxytoluene ([BHT] 20 $\mu\text{mol/l}$ in ethanol, -70°C) until analysis [11].

Plasma glucose and lipid analysis. Plasma glucose was measured with an enzymatic ultra violet test, using an HK/G6P-DH method (Cobas Fara Centrifugal analyser, Roche Diagnostic Systems, Welwyn Garden City, UK). Plasma cholesterol and triglyceride concentrations were determined using an enzymatic colorimetric test with the *in vitro* diagnostic reagent systems Unimate CHOL' (using cholesterol oxidase) and Unimate TRIG' (using glycerol phosphate dehydrogenase) (Cobas Fara). Plasma high-density lipoprotein (HDL)-cholesterol was determined enzymatically using the RANDOX method based on enzymatic addition of cholesterol esterase and cholesterol oxidase after buffering of LDL, VLDL and chylomicrons (Cobas Fara). Non-esterified fatty acids (NEFA) were quantified using a Wako NEFA C test kit (Alpha laboratories, London, UK) based on an acyl-coA synthetase/acyl coA oxidase enzymatic colorimetric method. LDL-cholesterol is present at very low concentrations in these rats [24] and measurements were not performed.

Probucol and 8-epi-PGF $_2\alpha$ analysis in plasma and tissue. Individual aortae provided insufficient tissue for analysis. Therefore, aortae within each group were pooled and homogenised, then subdivided to provide three separate samples for extraction and subsequent evaluation of probucol and 8-epi-PGF $_2\alpha$. Liver samples were analysed individually. Tissue samples were homogenised in ice-cold chloroform/methanol solution (2/2 v/v with BHT, 0.005 %). Sodium chloride (0.9 %) was added.

ed and the lipid fraction isolated by chloroform extraction. After solvent evaporation, this fraction was subjected to hydrolysis (30 min, 40°C with potassium hydroxide, 1.0 mol/l) and the sample divided for probucol and 8-*epi*-PGF_{2α} analysis. Probucol was measured by high-performance liquid chromatography according to the method of Nourooz-Zadeh et al. [25]. Quantitative analysis of total 8-*epi*-PGF_{2α} was performed by gas chromatography-mass spectrometry (GC-MS) using negative-ion chemical ionisation [11].

Assessment of vascular function. The small intestine and intact mesentery were removed from the animal and placed in cold physiological salt solution (PSS) ([mmol/l]: 119 NaCl; 25 NaHCO₃; 5.5 D-glucose; 1.18 KH₂PO₄; 1.17 MgSO₄; 4.7 KCl; 2.5 CaCl₂; 0.026 EDTA, disodium salt). Third-order branches of the mesenteric arterial arcade were dissected free of connective tissue and mounted in pairs as ring preparations on a small vessel myograph capable of measuring isometric tension. The methods have been described in detail previously [2, 26]. Briefly, arteries were bathed in PSS, gassed with 5% CO₂/95% O₂ at 37°C (pH 7.45), and the passive tension and internal circumference determined. The arteries were then set to an internal circumference equivalent to 90% of that which would be experienced when relaxed in situ under a transmural pressure of 100 mmHg (the maximum active tension for the minimum resting tension is developed at approximately this circumference). Arteries were then subjected to a routine 'run-up' procedure in which a total of five contractions to noradrenaline (NA, 5 μmol/l), 125 mmol/l potassium solution (KPSS; PSS with equimolar substitution of KCl for NaCl) and 5 μmol/l NA in KPSS were performed. All arteries produced an active tension equivalent to 100 mmHg (13.3 kPa) or more, and thereby fulfilled predetermined criteria for viability. Concentration-effect curves were constructed to NA (0.1–10 μmol/l), with concentration increments at 2 min intervals. Following repeated washing and recovery (10 min) arteries were submaximally contracted with NA to achieve approximately 80% of the maximum response. Endothelium-dependent dilator function was assessed by addition of half log molar increments of ACh at 2 min intervals (10⁻⁹–10⁻⁵ mol/l). A similar protocol was followed to assess endothelium-independent vasodilatation, by determining relaxation to sodium nitroprusside (SNP 10⁻⁹–10⁻⁵ mol/l). All procedures were approved by the British Home Office (Project License: PPL 90/00764) and followed the "Principles of laboratory animal care" (NIH publication No.85-23, revised 1985).

Chemicals and drugs. Chemicals used in this study were: NA (Sanofi Winthrop Limited, Guildford, UK); ACh, SNP, citric acid, trisodium citrate, BHT, indomethacin, pentafluorobenzyl bromide and diisopropylethylamine (Sigma-Aldrich Co. Ltd., Poole, UK); STZ (gift from Dr. N. MacLeod, UpJohn Co. Kalamazoo, Mich., USA); PGF_{2α}-d₄ from Cayman Chemicals (Ann Arbor, MI, USA); Sep-Pak C-18 and aminopropyl (NH₂) cartridges from Waters Chromatography (Watford, Herts, UK); N, O-bis(trimethylsilyl) trifluoroacetamide from Pierce Chemical Company (Rockford, Ill., USA); simvastatin as 'Zocor' tablets (Merck Sharp & Dohme Ltd., Hoddersdon, Herts, UK) and probucol (C₃₁H₄₈O₂S₂) (Sigma-Aldrich Co. Ltd). All other chemicals were obtained from Merck Lt (Poole, UK) and all were of analytical grade. For the vascular protocols, all concentrations are expressed as final molar concentrations in the vessel chamber.

Data calculation and statistical analysis. All values are given as mean ± SEM. For vascular protocols tension is given as the active wall tension (mN/mm artery length) and the relaxation re-

sponses to ACh and SNP were calculated as a percentage of initial precontraction to NA. The negative log of the concentration of agonist required to produce 50% of the maximum response (pEC₅₀) to NA, ACh and SNP was calculated for each artery. Two arteries were used from each animal and mean values calculated. *n* refers to the number of animals used unless otherwise stated. pEC₅₀ was calculated for each experiment using non-linear regression, and the sigmoid equation of a curve fitting program (GraphPad; Software, San Diego, Calif., USA). Comparisons were made between mean pEC₅₀ values and maximum relaxation of all groups (InStat GraphPad) by ANOVA, using the Tukey multiple comparison post test. Probucol plasma and tissue concentrations in control and diabetic groups were compared using the unpaired Student's *t*-test and isoprostane measurements in all groups were compared by ANOVA using the Tukey multiple comparison post test (InStat GraphPad). *n* refers to the number of plasma or tissue samples measured (one from each animal unless otherwise stated), or to the number of extractions analysed from pooled samples in the case of aorta. Significance was assumed when *p* was less than 0.05.

Results

Animal weight and consumption of diet. Of the groups fed the standard diet, the diabetic animals gained less weight than controls (mean weight; diabetic standard-fed 276 ± 11, *n* = 9 vs control standard-fed 375 ± 11 g, *n* = 11; *p* < 0.001). There was no statistical difference between weights of probucol-fed (291 ± 11 g, *n* = 12), simvastatin-fed (266 ± 10 g, *n* = 8), or standard-fed diabetic rats. Similarly, no difference was observed between weights of control rats fed probucol (386 ± 9 g, *n* = 11) when compared to those fed a standard diet. Diabetic rats consumed more diet per day than controls when fed the standard diet (diabetic standard-fed 50.9 ± 3.9 vs control standard-fed 31.3 ± 0.8 g; *p* < 0.001), probucol diet (diabetic probucol-fed 59.9 ± 3.7 vs control probucol-fed 33.2 ± 0.8 g; *p* < 0.001) or the simvastatin diet (diabetic simvastatin-fed 57.6 ± 5.6 g vs control; *p* < 0.001). There was no difference in daily consumption among the diabetic rats fed probucol, simvastatin, or standard chow.

Plasma glucose. Plasma glucose concentration was significantly greater in diabetic standard-fed rats compared with control standard-fed rats (diabetic 38.4 ± 2.0, *n* = 10 vs control 10.3 ± 0.7 mmol/l, *n* = 11; *p* < 0.001). These values for plasma glucose concentrations of control rats are consistent with those reported in other studies from this laboratory of rats also killed by CO₂ inhalation and cervical dislocation [2, 3]. There was also a significant difference in plasma glucose concentration between diabetic probucol-fed and control probucol-fed rats (diabetic probucol-fed 40.1 ± 1.4, *n* = 13 vs control probucol-fed 11.0 ± 0.4 mmol/l, *n* = 11; *p* < 0.001). There was no significant difference in plasma glucose concentration of diabetic rats on standard diet, probucol-sup-

Fig. 1a–d. Plasma (a) cholesterol, (b) triglyceride, (c) HDL-cholesterol, and (d) non-esterified fatty acid (NEFA) concentration (mmol/l) in control probucol-fed rats; $n = 6$ (CP), control standard-fed rats; $n = 6$ (C), diabetic standard-fed rats; $n = 4$ (D), diabetic probucol-fed rats; $n = 8$ (DP), and diabetic simvastatin-fed rats; $n = 8$ (DS) on dietary supplementation for 4 weeks. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

plemented diet, or simvastatin-supplemented diet (diabetic simvastatin-fed 37.1 ± 2.0 mmol/l, $n = 8$).

Results of lipid analysis. Plasma concentrations of cholesterol (diabetic 1.57 ± 0.15 vs control 0.94 ± 0.10 mmol/l; $p < 0.001$) (Fig. 1a), triglycerides (diabetic 2.51 ± 0.51 vs control 1.20 ± 0.08 mmol/l; $p < 0.01$) (Fig. 1b) and NEFA (diabetic 0.48 ± 0.13 vs control 0.17 ± 0.07 mmol/l; $p < 0.05$) (Fig. 1d) were significantly increased in standard-fed diabetic rats ($n = 5$) when compared to standard-fed control rats ($n = 6$). Plasma cholesterol concentration was significantly decreased in probucol-fed diabetic rats (diabetic probucol-fed 1.13 ± 0.08 mmol/l, $n = 7$ vs diabetic standard-fed; $p < 0.05$) and in simvastatin-fed diabetic rats (diabetic simvastatin-fed 0.99 ± 0.06 mmol/l, $n = 8$ vs diabetic standard-fed; $p < 0.01$) when compared with standard-fed diabetic rats. Plasma triglyceride concentrations were similarly reduced by both probucol (diabetic probucol-fed 1.18 ± 0.19 mmol/l vs diabetic standard-fed rats; $p < 0.01$) and simvastatin (diabetic simvastatin-fed

0.86 ± 0.14 mmol/l vs diabetic standard-fed rats; $p < 0.001$). Although numerically lower, plasma NEFA concentration in the diabetic rats was not significantly decreased by probucol (diabetic probucol-fed 0.23 ± 0.02 mmol/l) or by simvastatin (diabetic simvastatin-fed 0.26 ± 0.02 mmol/l) when compared to standard-fed diabetic. Probucol-fed control rats ($n = 6$) showed no difference in plasma cholesterol (0.81 ± 0.06 mmol/l), triglyceride (0.94 ± 0.06 mmol/l), NEFA (0.12 ± 0.07 mmol/l) or HDL-cholesterol (0.92 ± 0.07 mmol/l) concentrations compared to standard-fed controls. Plasma HDL-cholesterol (Fig. 1c) concentration was not different in standard-fed diabetic (0.66 ± 0.07 mmol/l) and standard-fed control (0.84 ± 0.07 mmol/l) rats, nor was it different in probucol-fed diabetic (0.82 ± 0.08 mmol/l) or simvastatin-fed diabetic (0.83 ± 0.15 mmol/l) compared to standard-fed diabetic rats.

Tissue and plasma probucol content. Probucol was assayed and detected in the lipid fractions of plasma, liver and aortic tissue from both diabetic and control rats on the probucol-supplemented diet and was not measurable in control or diabetic animals fed the standard diet. There was a 95 % increase in plasma probucol concentration in diabetic compared with control probucol-fed rats (diabetic probucol-fed 11.27 ± 1.07 , $n = 7$ vs control probucol-fed 5.79 ± 0.67 μ mol/l, $n = 6$; $p < 0.005$) (Fig. 2a). Probucol content of the aorta ($n = 3$ pooled sample) was increased

Fig. 2a–b. Plasma (a) probucol concentration (μmol/l), and liver and aortic (b) probucol content (μg/g) of control probucol-fed (CP) and diabetic probucol-fed (DP) rats (1 % probucol-supplemented diet for 4 weeks). * $p < 0.005$, ** $p < 0.0005$

Fig. 3a–b. Plasma (a) 8-epi-PGF_{2α} concentration (nmol/l), and liver and aortic (b) 8-epi-PGF_{2α} content (ng/g) of control probucol-fed rats (CP), control standard-fed rats; $n = 6$ (C), diabetic standard-fed rats; $n = 4$ (D) and diabetic probucol-fed rats (DP) on 1 % probucol-supplemented diet for 4 weeks. ** $p < 0.01$, *** $p < 0.001$

by 140 % (diabetic probucol-fed 19.48 ± 0.78 vs control probucol-fed 8.13 ± 0.26 μg/g; $p < 0.0005$) (Fig. 2b) and liver probucol content was increased by 277 % (diabetic probucol-fed 25.84 ± 3.82 , $n = 13$ vs control probucol-fed 6.86 ± 1.38 μg/g, $n = 11$; $p < 0.0005$ Mann Whitney) (Fig. 2b) in probucol-fed diabetic when compared to probucol-fed control rats.

Plasma concentrations of 8-epi-PGF_{2α}. Plasma 8-epi-PGF_{2α} concentration was measured in standard-fed and probucol-fed control and diabetic rats. Plasma concentrations of 8-epi-PGF_{2α} (Fig. 3a) were numerically higher in standard-fed diabetic (7.26 ± 2.47 nmol/l, $n = 4$) when compared to standard-fed control rats (0.99 ± 0.78 nmol/l, $n = 6$) but this difference did not achieve statistical significance and probucol-supplementation did not significantly reduce the plasma concentration of 8-epi-PGF_{2α} in the probucol-fed diabetic rats (5.75 ± 1.09 nmol/l, $n = 6$). 8-epi-PGF_{2α} was significantly raised in plasma of probucol-fed control rats when compared to control (control probucol-fed 12.43 ± 46 nmol/l, $n = 6$ vs control standard-fed; $p < 0.01$).

Tissue content of 8-epi-PGF_{2α}. The 8-epi-PGF_{2α} content of both aorta and liver (Fig. 3b) was measured in standard-fed and probucol-fed control and diabetic rats. 8-epi-PGF_{2α} was significantly increased in aorta of diabetic standard-fed rats when compared with controls (diabetic standard-fed 25.76 ± 1.47 vs control standard-fed 13.57 ± 1.28 ng/g; $p < 0.01$). Dietary probucol supplementation in diabetic rats reduced aortic 8-epi-PGF_{2α} content to values similar to those of control standard-fed rats (diabetic probucol-fed 13.00 ± 1.64 ng/g vs diabetic standard-fed; $p < 0.01$). Conversely, probucol supplementation in control rats increased the aortic content of 8-epi-PGF_{2α} to values higher than those observed in the diabetic rats (control probucol-fed 28.64 ± 2.89 ng/g vs control standard-fed; $p < 0.01$).

Liver content of 8-epi-PGF_{2α} was significantly increased in diabetic rats when compared to control standard-fed rats (diabetic standard-fed 10.20 ± 2.77 , $n = 10$ vs control standard-fed 0.96 ± 0.16 ng/g, $n = 7$; $p < 0.001$). Probucol supplementation in diabetic rats ($n = 13$) significantly reduced liver 8-epi-PGF_{2α} con-

Fig. 4. Relaxation of small mesenteric arteries in response to increased concentrations of acetylcholine (ACh) in control standard-fed rats; $n = 8$ ($\circ$), control probucol-fed rats; $n = 9$ (∇), diabetic standard-fed rats; $n = 8$ ($\bullet$), diabetic probucol-fed rats; $n = 8$ ($\blacktriangledown$) or diabetic simvastatin-fed rats; $n = 8$ ($\blacksquare$) on dietary supplementation for 4 weeks

tent towards control standard-fed values (diabetic probucol-fed 1.52 ± 0.18 ng/g vs diabetic standard-fed; $p < 0.001$). There was no significant difference in liver 8-epi-PGF_{2 α} content between probucol-fed and standard-fed control rats (control probucol-fed 2.95 ± 0.55 ng/g, $n = 11$ vs control standard-fed).

Vascular function. Mean mesenteric arterial internal diameter was 300 ± 5 μ m ($n = 80$ arteries) and there was no statistical difference in artery diameter between groups. Precontracted arteries from both control ($n = 8$) and diabetic ($n = 8$) rats relaxed in a concentration dependent manner to ACh. As reported previously [2], mesenteric arteries from diabetic animals on the standard diet

demonstrated a significant reduction in sensitivity (pEC_{50}) to ACh when compared to controls (Table 1, Fig. 4). Relaxation to ACh was not different in probucol-fed diabetic rats when compared to standard-fed diabetic rats. ACh-induced relaxation of arteries from probucol-fed control rats was also not different to those of standard-fed control rats. Mesenteric arteries from probucol-fed control and diabetic rats showed similar responses to NA or SNP to those of standard-fed control and diabetic rats (Table 1, Fig. 4). Similarly, arteries from diabetic rats on simvastatin supplementation ($n = 8$) demonstrated a significant reduction in sensitivity to ACh compared with control rats on standard diet and there was no difference between pEC_{50} or maximum response of arteries from diabetic rats on simvastatin or standard chow (Table 1, Fig. 4). Responses to SNP were not altered by simvastatin supplementation.

Discussion

This study has investigated the relationship of oxidative stress and abnormal plasma lipid concentration to vascular dysfunction of small mesenteric arteries in the STZ-rat. Determination of the function of these small arteries provides important insight into the mechanisms contributing to abnormal resistance in small arteries and arterioles and hence to disturbed capillary blood flow, both of which are associated with the vasculopathies of diabetes [27]. Several previous studies have documented a defect in ACh-mediated endothelium-dependent relaxation in the STZ-rat and this agonist was also used in the present study, as a blunted ACh-induced relaxation seems to be indicative of a global defect in the endothelium [6, 28].

The abnormal lipid profile of the STZ-diabetic rats in this study was similar to that previously reported [16, 18, 24, 29]. Other studies have shown elevation [18] and oxidation [10] of LDL in STZ-diabetic rats

Table 1. Effects of probucol and simvastatin dietary supplementation on vascular responses to ACh, NA and SNP of small mesenteric arteries from STZ-diabetic and control rats

		Control standard-fed ($n = 8$)	Control probucol-fed ($n = 9$)	Diabetic standard-fed ($n = 8$)	Diabetic probucol-fed ($n = 8$)	Diabetic simvastatin-fed ($n = 8$)
NA	pEC_{50}	5.891 $\pm$ 0.061	5.742 $\pm$ 0.040	5.817 $\pm$ 0.031	5.842 $\pm$ 0.045	5.704^a $\pm$ 0.045
	Max	3.24 $\pm$ 0.195	3.020 $\pm$ 0.252	3.281 $\pm$ 0.245	3.381 $\pm$ 0.254	3.747 $\pm$ 0.184
ACh	pEC_{50}	7.541 $\pm$ 0.175	7.466 $\pm$ 0.180	6.763^a $\pm$ 0.172	6.693^b $\pm$ 0.112	6.754^a $\pm$ 0.078
	Max	17.0 $\pm$ 6.4	24.0 $\pm$ 10.1	20.9 $\pm$ 6.3	27.7 $\pm$ 6.3	16.0 $\pm$ 6.5
SNP	pEC_{50}	7.313 $\pm$ 0.290	7.434 $\pm$ 0.294	7.212 $\pm$ 0.196	6.620 $\pm$ 0.089	7.224 $\pm$ 0.424
	Max	19.6 $\pm$ 9.8	17.3 $\pm$ 4.4	35.0 $\pm$ 8.0	30.3 $\pm$ 3.8	35.5 $\pm$ 11.0

^a $p < 0.05$ Tukey compared to control standard-fed; ^b $p < 0.05$ Tukey compared to control probucol-fed.

Max refers to maximum tension (mN/mm) achieved to noradrenaline or maximum % relaxation to ACh or SNP. All values are expressed as mean $\pm$ SEM

and this reactive lipid has been proposed to contribute to endothelial damage, while excess fatty acids [30, 31] and elevated triglycerides [32] may also affect endothelial function. Despite the potential for lipid-induced endothelial damage, this study clearly demonstrated that dietary supplementation with the HMG Co-A reductase inhibitor simvastatin (0.01 %) reversed the hypercholesterolaemia and hypertriglyceridaemia of the diabetic rats but did not affect vascular endothelial function of small mesenteric arteries. Similarly, dietary supplementation with the antioxidant lipid-lowering agent probucol (1 %) significantly improved both oxidative stress and dyslipidaemia of the diabetic rats but failed to improve vascular function.

This is, to our knowledge, the first investigation of the effect of lipid-lowering therapy on resistance-sized arteries from diabetic animals, although beneficial actions of the lipid lowering drugs, taurine [14] and cholestyramine [15], have been reported in aortae of diabetic mice. These opposing results may reflect variance in the initial lipid profile between diabetic mouse and rat. Alternatively, differences in the relative contributions of NO, prostaglandins and endothelium-derived hyperpolarizing factor to endothelium-dependent dilatation in rat resistance and mouse conduit arteries may be responsible.

In this study, there was no abnormality of the noradrenaline response in the diabetic compared with the control rats which differs from a previous study from our laboratory in female Wistar rats [2] and may suggest some strain and gender difference in catecholamine responsiveness in diabetic rats.

While studies investigating the relationship between dyslipidaemia and vascular endothelial function in diabetes are relatively few, the response to lipid lowering therapy in other disease states has been more widely reported and generally shows improvement of vascular function in hypercholesterolaemic animals [19, 20, 33] and human subjects with atherosclerosis [21, 34–36]. However, two studies have reported that probucol did not improve forearm vascular responses to ACh [37] or delay the progression of femoral atherosclerosis [38] in hypercholesterolaemic patients, despite increasing the resistance of LDL to oxidation and reducing plasma LDL-cholesterol concentration. The lack of correction of the endothelial defect in the present study compared with that in non-diabetic hypercholesterolaemic animals, suggests that metabolic disturbances other than hypercholesterolaemia are more detrimental to vascular endothelial function in this animal model of diabetes.

As probucol has antioxidant as well as lipid lowering properties, probucol accumulation into, and antioxidant efficacy in, the lipid fraction of plasma and the vasculature was determined. Probucol was present in plasma, liver and aorta of probucol-fed

animals and consistently higher levels were observed in the diabetic rats compared to control. The 1.8-fold increase in the daily food consumption of the diabetic rats is unlikely to explain the 2.4 and 3.8-fold rise in probucol content of aorta and liver respectively. These data therefore suggest that tissue uptake of probucol may be enhanced in the STZ-diabetic rat.

The high plasma concentrations and raised aortic and liver content of 8-epi-PGF_{2α} confirmed our earlier observations of oxidative stress in the diabetic rats [23] and the reversal by probucol suggested that the drug is indeed an effective antioxidant. To our knowledge, no previous study has reported direct evidence for oxidative stress in the vasculature of diabetic animals. F₂-isoprostanes are initially formed by oxidative modification of arachidonic acid in phospholipid membranes [39, 40]. The observation that probucol-supplemented control rats exhibited greatly increased plasma concentration and aortic 8-epi-PGF_{2α} content was unexpected. One explanation might be that probucol has a direct effect on arachidonic acid metabolism, but if this were the case a similar increase in 8-epi-PGF_{2α} would be anticipated in control and diabetic animals. Despite the observation that the probucol-fed control rats had greater tissue 8-epi-PGF_{2α} content, vascular function was no different to that of standard-fed control rats, thus providing further evidence for a dissociation between lipid peroxidation and vascular function.

This study supports a recent investigation from this laboratory, which demonstrated that dietary supplementation of vitamins E and C failed to improve vascular function in diabetic rats despite reducing oxidative stress as measured by evaluation of 8-epi-PGF_{2α} [23]. In summary, although probucol and simvastatin successfully reversed dyslipidaemia in the STZ-diabetic rat, these drugs failed to inhibit the development of endothelial dysfunction in small mesenteric arteries. Whether these observations pertain to all vascular beds in this animal model remains to be evaluated. This study provides further evidence that oxidative stress and abnormal lipids are not key mediators of impaired vascular function in small arteries of this animal model of diabetes, and point to a critical role of hyperglycaemia per se which was unaffected by any of the dietary regimes.

Acknowledgements. The authors thank the British Heart Foundation for supporting this project (grant number: PG/94186), Professor Tony Mallet for access to the UMDS Mass Spectrometry Facility for isoprostane analysis, and to Mr Peter Lumm (Department of Chemical Pathology, St Thomas' Hospital) for lipid analyses.

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