

# Inhibitory Effects of Low-Density Lipoproteins From Men With Type II Diabetes on Endothelium-Dependent Relaxation

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- OBJECTIVES** The object of the present study is to determine whether native (n) low-density lipoprotein (LDL) isolated from men with type II diabetes and abnormal endothelial function inhibits endothelium-dependent relaxation more than n-LDL isolated from nondiabetic control subjects.
- BACKGROUND** Endothelium-dependent vasodilation is impaired in men with type II diabetes and this may result from qualitative rather than quantitative abnormalities of LDL.
- METHODS** Forearm blood flow responses to brachial artery infusions of acetylcholine (endothelium-dependent vasodilator) and nitroprusside (endothelium-independent vasodilator) were measured in 10 men with uncomplicated type II diabetes and 10 nondiabetic men of similar age and with similar plasma concentrations of LDL cholesterol. Native LDL was isolated by discontinuous density gradient ultracentrifugation using EDTA to prevent oxidation. Precontracted rabbit aortic ring bioassay was used to determine inhibitory properties of n-LDL on endothelium-dependent relaxation by measuring relaxation to acetylcholine (and nitroprusside) in the presence and absence of n-LDL.
- RESULTS** Forearm blood flow responses to acetylcholine but not nitroprusside were significantly impaired ( $p < 0.01$ ) in diabetic men compared with control subjects. Native LDL (10 and 100  $\mu\text{g}$  protein/ml) from diabetic men inhibited relaxation to acetylcholine by  $13.9 \pm 4.8\%$  and  $61.9 \pm 7.8\%$  (mean inhibition for all doses  $\pm$  SE), respectively, whereas n-LDL from control subjects inhibited relaxation by  $7.3 \pm 3.0\%$  and  $23.9 \pm 5.7\%$  ( $p < 0.01$  for a difference between diabetic and control n-LDL). Relaxation to nitroprusside was not significantly inhibited by n-LDL.
- CONCLUSIONS** A qualitative abnormality of LDL may account for endothelial dysfunction in men with type II diabetes. (J Am Coll Cardiol 2000;35:1622-7) © 2000 by the American College of Cardiology

Atherosclerosis, the major cause of mortality in type II (noninsulin-dependent) diabetes mellitus, is initiated by injury to the endothelium (1). Low-density lipoprotein (LDL) is thought to initiate such injury (1) and in hypercholesterolemic subjects, dysfunction of the endothelium is evident as impaired endothelium-dependent vasodilation before the development of atherosclerosis (2). Endothelial function is impaired in patients with type II diabetes even in the absence of elevated LDL cholesterol (3,4). Physical characteristics of LDL particles are altered in type II diabetes and it has been suggested that this renders them more atherogenic (5,6). Whether LDL from diabetic sub-

jects is more injurious to the endothelium is, however, unknown. The purpose of the present investigation was to determine the effect of native (n) LDL isolated from men with type II diabetes, in whom endothelial function measured in vivo is abnormal, on endothelium-dependent vasorelaxation. To do this, we measured endothelial function in vivo, isolated n-LDL from diabetic and healthy control subjects and assessed its inhibitory effect on endothelium-dependent relaxation using a bioassay.

## METHODS

Men with type II diabetes ( $n = 13$ ), diagnosed on the presence of symptoms plus fasting glucose levels  $>7.0$  mmol/liter, were recruited from the Department of Endocrinology and Diabetes at St. Thomas' Hospital. The conditions of all diabetic men had been diagnosed at least three months previously and were controlled by diet or by

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**Abbreviations and Acronyms**

- ANOVA = analysis of variance
- HDL = high-density lipoprotein
- LDL = low-density lipoprotein
- n-LDL = native LDL
- NO = nitric oxide
- TBARS = thiobarbituric acid-reactive substances

diet plus oral hypoglycemic therapy (5/13). None had clinical evidence of atherosclerosis or of microvascular complications other than mild background retinopathy. All had urinary albumin/creatinine ratios within the normal range, and none had evidence of neuropathy on vibration testing. None were taking drugs other than oral hypoglycemic agents. Control men (n = 12) were recruited from hospital staff. In vivo measurements of endothelial function and in vitro effects of isolated LDL were each studied in 10 diabetic and 10 control subjects (some subjects were not available for both measurements because timing was constrained by the need to use freshly prepared LDL). The study protocol was approved by St Thomas' Hospital Research Ethics Committee and all subjects gave written, informed consent. Subject characteristics are shown in Table 1.

**Lipid profiles and LDL subfractions.** Serum total cholesterol, triglycerides and high-density lipoprotein (HDL) cholesterol levels were measured using standard enzymatic methods. Levels of LDL cholesterol were calculated using the Friedwald equation. The LDL subfractions in serum

frozen at the time of the study were later separated using high resolution polyacrylamide gel electrophoresis (Lipoprint LDL system; Quantimetrix; Redondo Beach, California) as previously described (7). This method identifies seven LDL subfractions (LDL-0 to LDL-6) of increasing density according to their relative electrophoretic mobility. The major subfraction was identified as the most intense band in a densitometric scan of the gel. In addition, the subfraction distribution was quantitated using an LDL score in which subfraction band (0 to 6) was multiplied by the relative contribution of each subfraction to the total. This score takes into account the relative proportions of each subfraction, providing an index of LDL density, a greater score being associated with smaller higher density LDL (7).

**Endothelial function in vivo.** Forearm blood flow studies were performed in a quiet clinical laboratory (temperature controlled to between 24°C and 26°C during each study) in the morning after a light breakfast. Treatment with oral hypoglycemic drugs was omitted on the morning of the study. The brachial artery was cannulated, and blood flow was measured by venous occlusion strain gauge plethysmography (8) during infusion of 0.9% saline or drugs dissolved in saline at 1.0 ml/min. Following determination of basal flow, blood flow was measured during infusions of three cumulative doses (7.5, 15 and 30 µg/min) of acetylcholine chloride (Coopervision; Southampton, United Kingdom) and, after blood flow had returned to baseline, of sodium nitroprusside (1, 3 and 10 µg/min, Roche; Basle, Switzerland). Each dose was infused for 6 min with blood flow (mean of five venous occlusions) measured during the last 2 min of each infusion period. Blood pressure was measured in supine position in triplicate using a vital signs monitor (Dinamap model 1846 SX; Critikon; Tampa, Florida) at the end of the study.

**Preparation and characterization of LDL.** Venous blood was collected into specimen tubes (Vacutainers) containing EDTA. Plasma was separated by low speed centrifugation at 4°C and n-LDL (density, 1.019 to 1.063 g/ml) then isolated from the plasma by discontinuous density gradient ultracentrifugation (9). Isolated n-LDL was then dialyzed with continuous stirring at 4°C for 24 h against two changes of 10 mM phosphate buffer solution (pH 7.4). The protein concentration was measured (10), and the final concentration of LDL was expressed as µg protein/ml. Freshly prepared LDL was used within 24 h for the isolated rabbit aortic ring studies described below. Oxidative modification of LDL was prevented by the inclusion of 0.3 mM EDTA in all buffers (11). Oxidation of LDL was sought by measurement of thiobarbituric acid-reactive substances (TBARS) (12). In each sample of isolated LDL, TBARS were below the limit of detection, being <1% of the value obtained when the sample was oxidized by exposure to Cu<sup>++</sup> (CuSO<sub>4</sub>, 1.7 µM) for 3 h.

**Table 1.** Subject Characteristics

	Control Subjects (n = 12)	Type II Diabetics (n = 13)
Age (yr)	50 (9)	47 (11)
BMI (kg·m <sup>-2</sup> )	27 (8)	27 (5)
Systolic BP (mm Hg)	127 (12)	133 (15)
Diastolic BP (mm Hg)	76 (14)	80 (8)
Glucose (mmol/liter)	4.8 (1.3)	9.2 (4.7)*
HbA <sub>1c</sub> (%)	5.1 (0.4)	7.7 (2.4)*
Total cholesterol (mg/dl)	213 (34)	182 (35)
HDL cholesterol (mg/dl)	58 (19)	39 (8)*
Triglycerides (mg/dl)	151 (86)	177 (106)
LDL cholesterol (mg/dl)	112 (8)	124 (12)
Major LDL subfraction†		
Band 0	5/10	4/10
Band 1	4/10	5/10
Band 2	1/10	1/10

\*p < 0.01 compared with control subjects.

†The LDL subfractions measured in 10 control subjects and 10 diabetics in whom LDL was isolated for in vitro studies.

BMI = body mass index; BP = blood pressure; HbA<sub>1c</sub> = glycosylated hemoglobin; HDL = high density lipoprotein; LDL = low-density lipoprotein.

**Bioassay to determine inhibitory effects of isolated LDL on endothelium-dependent relaxation.** New Zealand white male rabbits (2 to 2.5 kg) were sacrificed to obtain the descending thoracic aorta, which was trimmed of adhering tissue and fat. Paired transverse 2-mm-wide rings were cut and mounted in 3-ml organ baths containing oxygenated Krebs' solution (+ 0.3 mM EDTA), at 37°C. Tissues were placed under 2g resting tension for 60 min and tension adjusted to 2g for a further 30 min. Isometric measurements were recorded via force transducers (Grass FT03; Grass Inst; West Warwick, New York). Tissues were contracted with increasing doses of norepinephrine (0.06 to 0.12  $\mu$ M) to determine a concentration that gave 80% maximum contraction. Repeated exposure to this concentration was performed to establish that reproducible contractions were obtained. Relaxation dose-response curves to acetylcholine chloride ( $10^{-8}$  to  $10^{-6}$  M) were then obtained. Paired rings were incubated with either patient or control n-LDL at two concentrations of 10  $\mu$ g protein/ml and 100  $\mu$ g protein/ml for 20 min; contraction/relaxation was then repeated. Washout and relaxation of precontracted rings to acetylcholine were repeated between and after the two concentrations of n-LDL to ensure that baseline responses were maintained. Finally, after further washout, endothelium-independent relaxation to nitroprusside was examined in the presence and absence of n-LDL (100  $\mu$ g/protein/ml). The concentrations of LDL used were chosen to be similar to those that have been shown to have minimal inhibitory effects on endothelium-dependent relaxation in this preparation, when LDL is isolated from healthy volunteers (11).

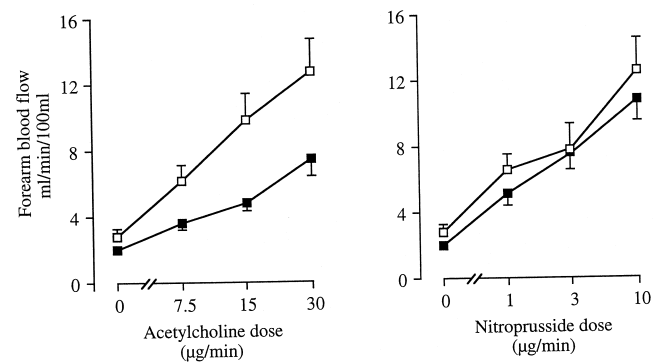
**Data analysis and statistical methods.** Descriptive statistics (Table 1) are summarized as means  $\pm$  SD. Results are expressed as means  $\pm$  SE. Analysis of variance (ANOVA) for repeated measures was used to test for differences in forearm vasodilator responses and for an interaction between the dose response and group. Blood flow data were log transformed prior to statistical analysis since the distribution of values thus transformed more closely approximated a normal distribution. Repeated measures ANOVA was also used to test for differences in inhibitory properties of isolated n-LDL on endothelium-dependent relaxation. In addition, the following summary measure of the inhibitory effects of LDL on relaxation (R) was calculated from the sum of the relaxations in the absence ( $\Sigma R$ ) and presence of LDL ( $\Sigma R_{LDL}$ ) to all doses of drug:

$$[(\Sigma R - \Sigma R_{LDL})/\Sigma R] \times 100\%.$$

Differences were considered significant if  $p < 0.05$ .

## RESULTS

**Lipid profiles and distribution of LDL subfractions.** Serum concentrations of total cholesterol, LDL cholesterol and triglycerides were similar in control and diabetic men. High-density lipoprotein cholesterol level was significantly lower in diabetic men than in control subjects (Table 1).

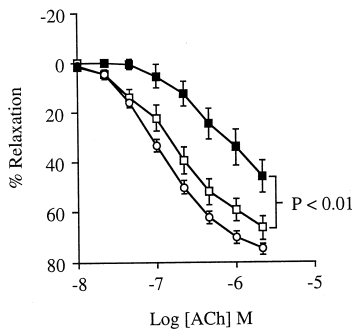


**Figure 1.** Forearm blood flow during brachial artery infusion of acetylcholine (7.5, 15 and 30  $\mu$ g/min) and nitroprusside (1, 3 and 10  $\mu$ g/min) in healthy control men (n = 10, open squares) and men with type II diabetes (n = 10, closed squares).  $p < 0.01$  for the comparison of blood flow responses to acetylcholine in men with type II diabetes compared with those in control subjects by ANOVA for repeated measures.

The distribution of major LDL subfractions was similar in diabetic and control men (Table 1). The LDL subfraction score was also similar in the two groups ( $1.7 \pm 0.2$  vs.  $1.6 \pm 0.2$  for diabetic and control subjects, respectively).

**Endothelial function in vivo.** During each blood flow study, blood flow in the noncannulated control arm did not change significantly in response to drug infusions in the contralateral arm, indicating that, at the doses used, these drugs had no systemic effects. Mean blood flow in the cannulated arm during infusion of acetylcholine and nitroprusside is shown in Figure 1. Blood flow responses to acetylcholine were significantly lower in men with type II diabetes compared with control men (Fig. 1,  $p < 0.01$ ). The highest dose of acetylcholine increased forearm blood flow to  $12.7 \pm 1.9$  ml/min per 100 ml forearm in control men and to  $7.4 \pm 1.0$  ml/min/100 ml in men with type II diabetes. Blood flow responses to nitroprusside were similar in control and diabetic men.

**Inhibitory effects of isolated LDL on endothelium-dependent relaxation.** The effect of n-LDL (100  $\mu$ g protein/ml) from the diabetic and control subjects on acetylcholine induced relaxation of the precontracted rabbit aortic ring bioassay is shown in Figure 2. The n-LDL from diabetic subjects inhibited relaxation to a greater extent than did n-LDL from control men ( $p < 0.01$  by ANOVA for repeated measures). The n-LDL (10 and 100  $\mu$ g protein/ml) inhibited relaxation to acetylcholine in a dose-dependent manner ( $p < 0.01$ , Fig. 3), with n-LDL from men with type II diabetes being significantly more potent than n-LDL from control men (percent inhibition:  $61.9 \pm 7.8\%$  vs.  $23.9 \pm 5.7\%$  for 100  $\mu$ g protein/ml n-LDL,  $p < 0.01$ , Fig. 3). Relaxation to nitroprusside was not significantly inhibited by n-LDL from control or diabetic men (Fig. 4). There was an inverse correlation between the in vivo response to acetylcholine (mean for all doses) and the

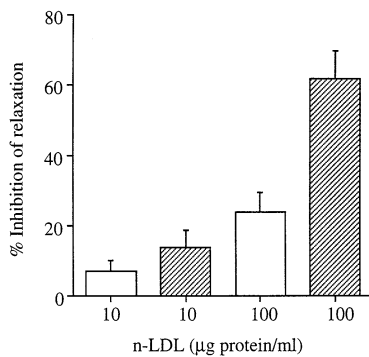


**Figure 2.** Bioassay system to examine effects of n-LDL isolated from men with type II diabetes (n = 10, **closed squares**) and control subjects (n = 10, **open squares**) on endothelium-dependent relaxation to acetylcholine (ACh) in rabbit aortic rings precontracted with noradrenaline. Relaxation in absence of n-LDL is shown in open circles. p value refers to comparison of inhibitory effects of n-LDL from men with type II diabetes with those of n-LDL from control subjects by ANOVA for repeated measures.

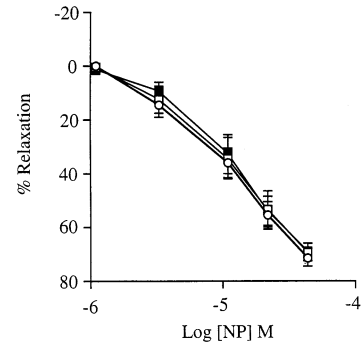
degree of impairment (mean inhibitory effect for both concentrations of LDL) of endothelium-dependent relaxation in vitro produced by isolated LDL (R = 0.57, p < 0.05).

**DISCUSSION**

Our findings that vasodilator responses to brachial artery infusion of acetylcholine are impaired in men with type II diabetes are in line with those of other investigators (3,4). Responsiveness to nitroprusside was not impaired. Vasodilator effects of acetylcholine in this vascular bed are mediated, at least in part, through the stimulation of the L-arginine/nitric oxide (NO) pathway within vascular endothelium (13). Those of nitroprusside, which degrades spontaneously to act as an NO donor or related vasodilator,



**Figure 3.** Inhibitory effects of n-LDL isolated from control men (n = 10, **open bars**) and men with type II diabetes (n = 10, **shaded bars**) on relaxation of rabbit rings to acetylcholine. Percent inhibition refers to the difference between the sum of relaxations to all doses to acetylcholine in the absence and presence of n-LDL. p < 0.01 for the difference between effects of n-LDL from controls and diabetics.



**Figure 4.** Effects of n-LDL isolated from men with type II diabetes (n = 10, **closed squares**) and control subjects (n = 10, **open squares**) on endothelium-independent relaxation to sodium nitroprusside in rabbit aortic rings precontracted with noradrenaline. Relaxation in absence of n-LDL is shown in **open circles**.

are mediated by the same effector mechanism but do not involve elaboration of NO from the endothelium. The present in vivo findings are thus most readily explained by impaired formation or increased inactivation of endothelium-derived NO in men with type II diabetes compared with healthy control subjects. Some (3,4) but not all (14) investigators have demonstrated impaired responses to the nitrovasodilators glyceryl trinitrate or nitroprusside in type II diabetes, in addition to impaired responsiveness to acetylcholine. It is possible that these variable findings with regard to nitrovasodilators relate to the characteristics of the diabetic subjects particularly with regard to the duration, control and presence of complications such as peripheral neuropathy (15).

Previous in vitro studies have shown that n-LDL impairs endothelium-dependent relaxation (11). In the present study, we have examined the possibility that n-LDL from men with type II diabetes is more potent in this regard than that from control subjects. The major novel finding of the study is that n-LDL from patients with type II diabetes produced markedly greater inhibition of endothelium-dependent relaxation to acetylcholine in a bioassay system than did equal concentrations of n-LDL isolated from control subjects. This suggests that a qualitative abnormality of n-LDL is responsible, at least in part, for the endothelial dysfunction seen in men with type II diabetes. By isolating n-LDL and studying its effects ex vivo, we have distinguished effects of LDL from those of other lipoproteins (the concentrations of which are often altered in type II diabetes). This study, therefore, provides direct evidence that the characteristics of LDL in type II diabetes are such as to increase its potency in inhibiting endothelium-dependent relaxation. Furthermore, since endothelial dysfunction is thought to precede atherosclerosis (1,16), the results strongly support the hypothesis that LDL in type II diabetes is more atherogenic than is LDL from healthy nondiabetic men. An interference supported by results from the 4S study (17) is that patients with type II diabetes may benefit to an

even greater degree than nondiabetic subjects from reductions in LDL cholesterol and that this benefit may extend to diabetic patients with LDL cholesterol levels within the conventional normal range. Furthermore, there may be scope for interventions that modify the characteristics of n-LDL to decrease its inhibitory actions on the endothelium and hence its atherogenicity. Qualitative differences in the injurious effects of LDL on the endothelium may exist in non diabetic patients (for example, those with insulin resistance) and may explain why, in patients with otherwise similar risk factors, prediction of cardiovascular events from the concentration of LDL cholesterol alone is of limited precision.

There are a number of characteristics of LDL that are altered in type II diabetes and that might potentially account for its increased inhibitory actions on the endothelium. These include the distribution of LDL in smaller denser subfractions and the oxidation, glycosylation and desialylation of LDL. The importance of small dense LDL has received much attention because of experimental evidence suggesting increased atherogenic potential of small dense LDL and evidence, mainly in nondiabetic subjects, that small dense LDL is associated with a higher incidence of ischemic heart disease (6,18). In the present study, we found that the LDL subfraction distribution and concentrations of triglycerides (closely related to LDL size) were not significantly different in diabetic and control men. Thus, although we did not measure absolute particle size, it is unlikely that this differed in the diabetic and nondiabetic men in this study. This is consistent with previous studies in which the association of type II diabetes with LDL particle size has been largely restricted to women, with only a weak or nonsignificant association seen in men (19). It is unlikely, therefore, that the increased inhibitory effects on endothelium-dependent relaxation of the LDL from diabetic men in this study relates simply to altered LDL particle size. Previous measurements of endothelial function in vivo from our laboratory also suggest that particle size is not the main determinant of endothelium-dependent vasodilation in nondiabetic men. In a series of 50 men (24 studied in previously published studies [2,20]) with a range of serum concentrations of LDL cholesterol of 72 to 240 mg/dl and triglycerides of 57 to 698 mg/dl, we observed a strong correlation between the vasodilator response to acetylcholine and total cholesterol ( $R = 0.44$ ,  $p < 0.01$ ), but no independent correlation with triglyceride concentration.

Oxidative stress is increased in type II diabetes and could result in partial oxidation of LDL, which is known to alter its properties in inhibiting endothelium-dependent relaxation (11). In the present study, LDL was collected into EDTA to prevent oxidation, and TBARS were not detectable. The presence of any substantial degree of oxidation, particularly that occurring *ex vivo*, was thus excluded. An electronegative subfraction of LDL (LDL<sup>-</sup>) has been identified in plasma that exhibits minor degrees of oxidation and is cytotoxic to endothelial cells (21). Concentrations of

circulating LDL<sup>-</sup> may be elevated in type II diabetes (5), possibly contributing to the increased inhibitory effects on endothelium-dependent relaxation. Low-density lipoprotein in patients with diabetes may be glycosylated and may contain less sialic acid than that from nondiabetic subjects (5). Increased inhibitory actions of diabetic LDL on endothelium-dependent relaxation may result from a combination of altered physico-chemical properties such as an increased proportion of electronegatively charged, glycosylated and desialylated particles. Further studies will be required to relate such properties of LDL to the impairment of endothelium-dependent relaxation.

In conclusion, we have demonstrated that LDL isolated from men with type II diabetes and endothelial dysfunction is more potent in inhibiting endothelium-dependent relaxation than that isolated from control subjects. Qualitative differences in the ability of LDL to injure the endothelium may contribute to endothelial dysfunction and the increased prevalence of atherosclerosis in type II diabetes. Interventions to modify the characteristics of LDL may be of importance in restoring endothelial function in type II diabetes. Patients with type II diabetes may also benefit from reduction of LDL cholesterol levels below the targets set for nondiabetic patients.

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