Impaired endothelium-dependent and independent dilatation of forearm resistance arteries in men with diet-treated non-insulin-dependent diabetes: role of dyslipidaemia

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1. We measured endothelium-dependent and independent dilatation of forearm resistance arteries in 29 men with diet-treated non-insulin-dependent diabetes mellitus and 18 age- and sex-matched control subjects. None of the diabetic patients had hypercholesterolaemia, overt hypertension or microproteinuria.

2. We examined endogenous and exogenous nitric oxide-mediated vasodilatation by measuring forearm blood flow with venous occlusive plethysmography after administration of acetylcholine (7.5 and 15 µg/min) and sodium nitroprusside (3 and 10 µg/min), respectively, into the brachial artery. L-NAME, NG-monomethyl-L-arginine was also infused to study the inhibition of basal and stimulated release of nitric oxide.

3. The vasodilatory response to acetylcholine, expressed as area under curve, was significantly decreased in the diabetic patients compared with the control subjects (P = 0.019). L-NAME-monomethyl-L-arginine significantly reduced basal (P < 0.001) and acetylcholine-stimulated blood flow (P < 0.02) in both groups. The vasodilatory response (also expressed as area under curve) to sodium nitroprusside was significantly less (P = 0.044) in the diabetic patients than in the control subjects.

4. In the diabetic patients, impaired vasodilatory responses to acetylcholine were significantly correlated with higher serum triacylglycerols (P = 0.048) and lower high-density lipoprotein-cholesterol concentrations (P = 0.007); the association with high-density lipoprotein was independent of age, glycated haemoglobin and blood pressure. Sodium nitroprusside responses were not correlated with lipid and lipoprotein concentrations.

5. We conclude that there is impaired endothelial and smooth muscle cell function in men with diet-treated non-insulin-dependent diabetes mellitus uncomplicated by overt hypertension or microproteinuria. Endothelial dysfunction may be related to diabetic dyslipidaemia and associated metabolic disturbances.

INTRODUCTION

Vascular disease is the major complication of non-insulin-dependent diabetes mellitus (NIDDM) [1, 2]. The pathogenesis of diabetic vasculopathy is poorly understood, but may involve endothelial cell dysfunction resulting in impaired release and action of endothelium-derived vasoactive factors, in particular nitric oxide (NO) [3, 4]. Endothelial dysfunction in NIDDM may not only be causally related to atherosclerosis [4, 5], but also to the development of hypertension [6], microangiopathy [7] and cardiomyopathy [8].

Experimental evidence supports the notion that endothelial dysfunction occurs in diabetes [3, 9]. Several metabolic abnormalities seen in human diabetic patients may independently and collectively impair the physiology of NO [3, 9]. These include increased glycation of plasma and cellular proteins, increased oxidative stress, insulin resistance and dyslipidaemia. Elevated serum concentrations of triacylglycerols with low high-density lipoprotein (HDL)-cholesterol and normal total cholesterol concentrations is the typical lipid phenotype seen in NIDDM [10]. In epidemiological studies, so called diabetic dyslipidaemia has been shown to predict cardiovascular disease [11], but its role in the early development of endothelial dysfunction has not yet been explored [5]. Diabetic dyslipidaemia is associated with increased production of small, dense low-density lipoprotein (LDL) [12] which has heightened susceptibility to oxidative modification [13]. Given that both oxidatively modified LDL and

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Key words: diabetes, dyslipidaemia, endothelium, nitric oxide, vasodilatation.
Abbreviations: ACh, acetylcholine; NIDDM, non-insulin-dependent diabetes mellitus; L-NAME, NG-monomethyl-L-arginine; NO, nitric oxide; SNP, sodium nitroprusside.
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low HDL can impair the production/action of NO and arterial vasomotor tone [5, 14, 15], it appears likely that dyslipidaemia may aggravate endothelial function in NIDDM.

Endothelium-dependent and independent vasodilatation may be studied in vivo by measuring basal and agonist-stimulated blood flow in forearm resistance arteries using venous occlusive plethysmography [16]. This model may reflect abnormal vessel wall function in other vascular beds [17]. Several studies have documented impaired NO-mediated vasodilatation in patients with atherosclerosis [18], hypercholesterolaemia [19] and hypertension [20]. Not all studies concur that NO-mediated vasodilatation is impaired in insulin-dependent diabetes [21, 22]. Two formal reports have been published on patients with NIDDM showing abnormalities in both endothelium-dependent and independent vasodilatation [23, 24]. Because these studies included patients with proteinuria [25], hypoglycaemic agents [26], post-menopausal women [27] and autonomic neuropathy [28], it was not strictly possible to infer that the vascular abnormalities were attributed to the diabetic state itself or to associated metabolic disturbance. Also, the effect of dyslipidaemia could not be adequately examined.

In the present study, we have employed the isolated forearm model to investigate endothelium-dependent and independent vasodilatation of forearm resistance arteries in diet-treated men with NIDDM uncomplicated by marked hypercholesterolaemia, clinical hypertension or microproteinuria. We hypothesized that the presence of dyslipidaemia would be associated with endothelial function.

PATIENTS AND METHODS

Patients

We studied 29 Caucasian men with diet-treated NIDDM recruited from the general community, and 18 age-and sex-matched healthy control subjects. Recruitment was by response to a newspaper advertisement. All patients had glycated haemoglobin (HbA1c) <11%, and were normotensive (supine blood pressure <145/90 mmHg) [29]. Patients were excluded if they had a fasting serum cholesterol level >6.5 mmol/l due to LDL-cholesterol [30], urinary albumin/creatinine ratio >3 mg/mmol [31], autonomic neuropathy [32], an ankle/arm blood pressure index ≤1, and a history of cardiovascular disease or any major systemic illness. All participants were non-smokers, and none was taking antioxidant vitamins, fish oil supplements, aspirin, antilipaemic drugs or antihypertensive medication. Retinopathy was assessed by direct ophthalmoscopy, and neuropathy by eliciting ankle reflexes and measuring vibration perception threshold at both ankles and toes. Forearm length was recorded. Every subject gave informed consent and the study was approved by the Royal Perth Hospital Ethics Committee.

Protocol

All participants were studied in the morning after fasting for 12 h. Studies were carried out in a purpose-designed vascular laboratory (ambient temperature 26–28°C). Resting blood pressure was measured by an automated blood pressure recorder (Dinamap model 845XT; Critikon, FL, U.S.A.), after resting quietly in the supine position for 10 min, at 2 min intervals over 24 min; the first two measurements were discarded and the mean of ten measurements was recorded. Forearm blood flow was measured with the subjects lying supine by venous occlusive plethysmography (Hokanson Inc., Bellevue, WA, U.S.A.) using mercury-in-silastic strain gauges [33]. During measurement wrist cuffs were inflated to 200 mmHg to fully occlude blood flow to the hands for approximately 1 min, and upper arm cuffs were inflated to 40 mmHg to occlude venous return for 10 s and deflated for 7 s for four cycles per measurement. The timing was selected to ensure that the forearm blood flow returned completely to its basal level before the next cycle commenced. Measurements of forearm blood flow were recorded on a MacIntosh PC (Cupertino, CA, U.S.A.) using the MacLab 3.3 (ADI Instruments, Sydney, Australia) computerized chart recorder system. The brachial artery of the non-dominant arm was cannulated with a 22-gauge plastic cannula under local anaesthetic (2% procaine HCl) and flushed with normal saline. Normal saline (0.9% NaCl) was infused for 15 min at a rate of 1 ml/min, followed by 5 min each of sodium nitroprusside (SNP) at 3 and 10 μg/min, acetylcholine (ACh) at 7.5 and 15 μg/min, and then N⁶-nmonomethyl-L-arginine (l-NMMA) at 1 mg/min for 8 min followed by a co-infusion with ACh at 15 μg/min for 5 min. Seventeen control and 27 diabetic subjects completed the l-NMMA protocol. The SNP, ACh and l-NMMA infusions were interspersed with infusions of normal saline for at least 10 min or until steady-state basal blood flow was achieved. The final minute of measurements from each dose of infusion was used for analysis. Basal forearm blood flow was expressed as ml blood flow min⁻¹ 100 ml⁻¹ forearm [33] and stimulated flow was calculated as:

\[
(\frac{I_d}{NI_d} - \frac{I_e}{NI_e}) \times \frac{(I_e/NI_e)}{(I_d/NI_d)} \times 100\%
\]

where \( I \) and \( NI \) represent forearm blood flow in the infused and non-infused arms, respectively, during the period of drug (d) and saline (s) administration. This method of calculation controls for extraneous factors influencing forearm blood flow [34]. The overall blood flow response to each drug (SNP, ACh) was calculated as the area under the curve (AUC). Forearm vascular resistance was calculated...
as mean arterial blood pressure divided by forearm blood flow.

**Laboratory methods**

Plasma glucose was determined using an automated glucose oxidase method (Technicon). Glycated haemoglobin (HbA1c) was measured by quantitative electrophoresis. Serum cholesterol and triacylglycerols were measured enzymically using an automated analyser (Cobas Mira; F. Hoffman-La Roche and Co.) with the Cholesterol Reagent (Trace Scientific), respectively. HDL-cholesterol was measured after precipitation of apolipoprotein B-containing lipoproteins with heparin/manganese. Non-HDL-cholesterol was calculated as (total cholesterol–HDL-cholesterol). Urinary albumin was measured by immunonephelometry using the Behring nephelometer system (Marburg, Germany). Urinary creatinine was measured on a Technicon Axon Analyser by the Jaffé reaction. The analytical imprecision for all biochemical assays was < 6%.

**Statistical methods**

The AUC of forearm blood flow ratio versus infusion doses of ACh and SNP was calculated by the trapezoid method using the Prism programme (Graphpad Software Inc., San Diego, CA, U.S.A.). Group comparisons were carried out by paired and unpaired t-test. Relationships between variables were assessed by simple and multiple linear regression methods. Statistical significance was defined at the 5% level. Skewed variables were log-transformed.

**RESULTS**

Table 1 shows the characteristics of the NIDDM patients and control subjects. The groups were well matched for age and serum cholesterol concentrations. Body mass index was higher in the diabetic patients, with overlap between groups. Glycaemic control in the patients was moderate-to-good. The diabetic patients had higher serum concentrations of triacylglycerols and non-HDL-cholesterol and lower levels of HDL-cholesterol than the control subjects. While blood pressure was slightly higher in the diabetic patients, none were hypertensive by WHO criteria [29]; blood pressure did not change significantly over the time of the infusion experiments. The mean duration of diabetes was 3.6 (range 1.0–10) years. Three of the 29 patients had mild background retinopathy (< 5 microaneurysms) and none had peripheral neuropathy (both ankle jerks present; normal vibration perception thresholds).

Figure 1(a) shows the increase in forearm blood flow ratio in response to 7.5 and 15 µg/min ACh in control and diabetic subjects. At both infusion rates, the diabetic patients had a significantly smaller increase in forearm blood flow ratio. Figure 1(b) shows the blood flow responses to 3 and 10 µg/min SNP in control and diabetic subjects, with a significantly impaired response in the diabetic patients at the higher dose of SNP. Correcting for forearm length did not alter the findings in Fig. 1. Mean forearm vascular resistance both at rest and in response to ACh was higher in the diabetic patients than in control subjects, but the difference was not statistically significant (P = 0.07).

Figure 2 shows the AUC of forearm blood flow response to ACh and SNP, expressed as % increase in blood flow ratio × dose range in µg/min, in the control and diabetic subjects. The AUC for the ACh response was significantly decreased by 52% in the diabetic patients compared with control subjects: 960 (347–3178) compared with 2005 (328–9070), P = 0.019. The AUC for the SNP response was also significantly diminished by 25% in the diabetic patients compared with control subjects: 2439 (556–5780) compared with 3259 (473–5614), P = 0.044. There was a significant positive correlation between the AUC response to ACh and SNP in the control subjects (r = 0.56, P = 0.02), but not in the diabetic patients (r = 0.31, P = 0.10). In analysis
of covariance, group assignment was the only significant correlate of vascular responses to ACh and SNP. Neither glycated haemoglobin, serum lipids and lipoproteins or blood pressure alone accounted for the differences between diabetic patients and control subjects.

Figure 3 shows that the response to ACh (15 µg/min) with the co-infusion of L-NMMA was significantly decreased ($P<0.05$) to the same extent in both control (119%) and diabetic (110%) subjects. Responses to the ACh/L-NMMA infusion were positively associated with responses to SNP (AUC) in the control subjects ($r = 0.59$, $P = 0.01$) but not the diabetic patients ($r = 0.19$, $P = 0.34$).

There was no significant difference in basal blood flow between control subjects and diabetic patients, mean (range): 2.08 (1.70–2.48) compared with 2.22 (1.77–2.69) ml min$^{-1}$ 100 ml$^{-1}$ forearm. This was in spite of resting blood pressure and fasting glucose being significantly increased in the diabetic patients (Table 1). After infusion of L-NMMA there was a similar reduction in the percentage blood flow in the control subjects [-25.4 (-58.7 to -6.0)%] and diabetic patients [-24.3 (-49.9 to -38.5)%], $P<0.001$ for each group. L-NMMA did not alter arterial blood pressure or heart rate significantly in diabetic patients or control subjects. Forearm vascular resistance increased comparably in both groups.

Table 2 shows the correlations between serum lipid and lipoprotein concentrations and the increase in forearm blood flow (AUC) with ACh in the diabetic patients. The response to ACh was positively correlated with HDL-cholesterol concentrations (Fig. 4a) and negatively correlated with triacylglycerol levels (Fig. 4b). The positive association between blood flow response and HDL-cholesterol was independent of age, systolic blood pressure and glycated haemoglobin (see Table 3). The correlations between stimulated blood flow and triacylglycerols and HDL-cholesterol were more significant at the lower than the higher dose of ACh. The AUC for SNP was not significantly correlated with serum lipid and lipoproteins in the diabetic patients. No significant correlations were seen between serum...
Table 2. Correlations between the percentage increase in forearm blood flow (AUC) in response to ACh and SNP and serum concentrations of cholesterol, non-HDL-cholesterol, HDL-cholesterol and triacylglycerol in 29 men with NIDDM. Pearson's correlation coefficients and P-values are shown.

<table>
<thead>
<tr>
<th>Serum lipids and lipoproteins</th>
<th>Forearm blood response</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ACh (AUC) P-value</td>
</tr>
<tr>
<td></td>
<td>r</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.055</td>
</tr>
<tr>
<td>Non-HDL-cholesterol</td>
<td>-0.064</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>0.492</td>
</tr>
<tr>
<td>Triacylglycerol</td>
<td>-0.370</td>
</tr>
</tbody>
</table>

Table 3. Multiple linear regression model predicting the increase in forearm blood flow with ACh (area under dose–response curve) in patients with NIDDM (n = 29). HDL-cholesterol is expressed as μmol/l. $R^2 = 22.5%$.

<table>
<thead>
<tr>
<th>Predictor variable</th>
<th>Regression coefficient</th>
<th>Standard error</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDL cholesterol</td>
<td>1.54</td>
<td>0.57</td>
<td>0.012</td>
</tr>
<tr>
<td>Age</td>
<td>-40.70</td>
<td>22.24</td>
<td>0.080</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>-2.73</td>
<td>11.92</td>
<td>0.821</td>
</tr>
<tr>
<td>Glycated haemoglobin</td>
<td>-124.14</td>
<td>124.92</td>
<td>0.331</td>
</tr>
</tbody>
</table>

DISCUSSION

We have demonstrated that diet-treated men with NIDDM have a dysfunction of both endothelial and smooth muscle cells in forearm resistance arteries and that the former is related to dyslipidaemia. The findings apply to patients without overt vascular disease, hypercholesterolaemia, hypertension, microalbuminuria or autonomic neuropathy, factors that may independently influence vasomotor function [18–20, 25, 28]. The results are compatible with the notion that NIDDM is associated with a disorder in NO-mediated vasodilatation [23–25, 35].

That endothelial dysfunction occurs in diabetes is supported by studies in animals [3, 6, 9]. We complement and extend previous reports in the forearm vascular bed in patients with NIDDM [23, 24]. These studies, however, included subjects with confounding characteristics, such as female gender, albuminuria, autonomic neuropathy, peripheral vascular disease and treatment with oral hypoglycaemic agents and insulin [18, 25–28]. Abnormal vasodilatory responses to exogenous nitrates were seen in the diabetic patients and, as in our study, no correlation was found with responses to a muscarinic agonist. Williams et al. [24] employed methacholine as the muscarinic agonist, but this agent may be less specific to the NO pathway than
ACh [36] and the effects of L-NMMA were not reported. McVeigh et al. [23] did not find that L-NMMA decreased basal blood flow in either diabetic patients or control subjects, but this might have been due to differences in patient characteristics and dose of L-NMMA employed [37]. Goodfellow et al. [38] recently reported abnormal flow-mediated dilatation of the brachial artery in a small group of diabetic patients, suggesting that our findings may extend to other vascular beds. Because of smaller sample sizes and confounding variables some of the aforementioned studies could not fully evaluate the association between dyslipidaemia and vascular dysfunction in NIDDM.

Various mechanisms may account for the abnormal vasomotor findings in NIDDM. A disturbance in the physiology of endothelium-derived NO may be due to decreased basal NO synthesis, to disturbed coupling of muscarinic receptors with NO synthase, or to increased inactivation of NO [4]. The fact that L-NMMA decreased basal forearm blood flow to the same extent in diabetic patients and control subjects argues against the first mechanism [37]. Since there were divergent abnormalities in the forearm responses to ACh and SNP, we cannot make definite inferences regarding alternative mechanisms. Increased oxidative stress in diabetes may affect both the biotransformation of exogenous nitrates and the inactivation of endogenously released NO [39]. ACh decreases vascular resistance by mechanisms other than via endogenous release of NO [40, 41], as evidenced by the partial inhibition of the response in both diabetic patients and control subjects with L-NMMA. The predominant muscarinic action of ACh, however, is to stimulate constitutive NO synthase in endothelial cells [4, 36]. ACh releases other vasodilators, such as prostacyclin [40] and endothelium-derived hyperpolarizing factor [41]. That forearm responses to a co-infusion of ACh and L-NMMA were similar in diabetic patients and control subjects confirms that in NIDDM the vascular defect is specific to the NO pathway. Williams et al. [24] recently showed that vasoconstrictor prostanooids and a generalized abnormality of vascular smooth muscles do not contribute significantly to vascular dysfunction in NIDDM. Further studies should employ agonists that operate through different signal transduction pathways and interventions that increase the bioavailability of NO.

Hyperglycaemia, hyperinsulinaemia, dyslipidaemia and increased oxidative stress are all possible biochemical mediators of vascular wall dysfunction in diabetes [3, 9, 10]. Experimental evidence indicating that hyperglycaemia mediates vessel wall abnormalities has not been consistently confirmed in humans [9], but our results might have been different had we studied patients with poorer glycaemic control [42]. Microvascular dysfunction also has been described in the pre-diabetic state [43], indicating the importance of insulin resistance. Hyperinsulinaemia in NIDDM reflects resistance to the physiological effects of insulin and it is possible that the vasomotor abnormalities in our patients were specifically related to resistance to NO-mediated actions of insulin [35, 44]. Hypertriglyceridaemia and low HDL-cholesterol were the only significant correlates of endothelial dysfunction in the present study. These metabolic variables are intimately correlated, the closer association of endothelial dysfunction with low HDL probably reflecting the greater variability in the measurement of serum triacylglycerol. While the triacylglycerol/HDL nexus may simply reflect insulin resistance [10], it is also associated with increased production of small, dense LDL particles [12]. These are highly susceptible to oxidative modification [13], particularly in the presence of a low HDL pool [14]. Human and animal studies support a role for oxidized LDL in endothelial dysfunction [5, 45], an effect that may be counteracted by HDL [15, 46]. A role for oxidative stress in the development of diabetic complications has been well emphasized [47]. We propose that this, together with increased production of small, dense LDL and low turnover of HDL, contributes to endothelial dysfunction in NIDDM.

Potential limitations of our study relate to the use of highly selected patients, the pharmacological protocol and the cross-sectional design. As discussed previously, we selected patients who would not confound our study hypotheses. Our sample size was larger than in previous reports and the prevalence of dyslipidaemia in the absence of overt hypercholesterolaemia was comparable to other unselected populations [10, 29]. We studied forearm resistance vessels since these do not develop atherosclerosis, which impairs NO-mediated vasodilatation directly [18]. As suggested elsewhere [8, 17, 38], however, our findings may extend to other vascular beds. Since we did not employ a NO-independent smooth muscle cell agonist, we cannot conclude whether the impaired response to SNP is due to increased degradation of NO by free radicals or to a generalized defect in smooth muscle cell function, although we consider the former more likely [24]. Finally, correlation analyses are not demonstrative of causality and controlled intervention studies are required to further explore the mechanism of diabetic vascular dysfunction.

Our results suggest that the pathogenesis of diabetic vascular complications may involve an interaction between dyslipidaemia and agonist-stimulated NO release from endothelial cells. This may not only result in abnormal vasomotor tone, but also in increased platelet aggregation, monocyte adhesion and smooth muscle cell proliferation [3, 4, 9]. These broader effects are particularly relevant to the development of diabetic macrovascular disease [1, 11]. It remains to be shown, however, whether the alterations in vasomotor tone that we have reported predict the development of both structural and clinical vascular abnormalities, and whether
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References


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Functional changes in resistance vessels may be reversed by lipid-modifying therapy.