Endothelium-Dependent and -Independent Vascular Dysfunction in Type 1 Diabetes

Role of Conventional Risk Factors, Sex, and Glycemic Control

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Objective—Defective NO release/response may contribute to increased coronary risk and the loss of sex difference in coronary heart disease in diabetes. We aimed to determine whether NO release/response is impaired in type 1 diabetes and whether any defects are greater in women than men.

Methods and Results—Forearm blood flow response to vasoactive drugs was assessed by venous plethysmography in 88 diabetic and 69 control subjects aged 30 to 53 years. In diabetic patients, response was 18% lower for acetylcholine (ACh) (P=0.002), 6% lower for bradykinin (P=0.14), and 17% lower for glyceryl trinitrate (GTN) (P<0.001). Women had a higher response to ACh than men (17%, P=0.006). The diabetes-associated defect in ACh was greater in women (25% lower, P=0.01) than men (13% lower, P=0.08), although not significantly (P=0.26 for the interaction). Poorer glycemic control was associated with ACh response (P=0.003) and contributed to the greater defect in diabetic women than men.

Conclusions—The diabetes-associated defect in GTN response was similar in men and women. Established coronary heart disease risk factors did not explain any of the defects in ACh or GTN response associated with diabetes. Type 1 diabetes is associated with impaired responsiveness to NO and with an impairment in ACh-stimulated NO release. (Arterioscler Thromb Vasc Biol. 2003;23:1048-1054.)

Key Words: nitric oxide □ endothelial dysfunction □ diabetes mellitus □ plethysmography

In type 1 diabetes, there is a 3-fold increase in coronary heart disease (CHD) mortality in men and a 7-fold increase in women.1 Hence, the sex difference in CHD mortality seen in the general population is abolished. Established CHD risk factors do not account fully for the increased CHD risk nor the loss of sex difference in risk in type 1 diabetes,2 and interest has focused on the potential role of the endothelium.

Endothelium-derived NO has several potential antiatherogenetic actions, including inhibition of platelet aggregation, adhesion molecule expression, and vascular smooth muscle cell proliferation,3 and defective production or activity of NO enhances atherosclerosis in experimental models.4 Various defects in NO pathways have been reported in type 1 diabetes in humans and animal models.5 However,6 the in vivo data are inconsistent, and it is not clear whether the defect is in basal or stimulated NO synthesis, NO bioavailability, responsiveness to NO, or perhaps all of these.5 Furthermore, the study sizes (at least in forearm resistance vessel studies) have tended to be small, subject selection has often been such that the results may not be generalizable to the typical diabetic population, and there are no data on sex differences in endothelial responses in type 1 diabetes. By far the largest examination of forearm resistance vessel reactivity in type 1 diabetes to date studied 50 diabetic patients and found no defect in stimulated or basal NO production.7

Thus, our study had 3 aims: to clarify whether there is a functional defect in the L-arginine/NO pathway in diabetes, to examine whether diabetes has a greater effect on endothelial function in women compared with men, and to examine whether the reported sex difference in NO-mediated dilatation is abolished in diabetes.

Methods

Subjects

Participants were recruited from a cohort of 400 subjects (199 type 1 diabetic and 201 nondiabetic subjects) who had taken part in a previous study assessing coronary risk factors.8 Type 1 diabetes was defined as diabetes with age of onset ≤25 years with continuous use of insulin within 1 year of diagnosis. Diabetic patients were a random sample from the diabetes registers of 5 London hospitals. Those with renal failure or undergoing renal replacement therapy were excluded, but otherwise the diabetic patients were deliberately sampled to be representative of the distribution of glycemic control, blood pressure, and other vascular risk factors in the type 1 diabetic population at this age. The nondiabetic participants were a random sample from the patient registers of two London general practices. Nondiabetic subjects were also sampled without regard to their history of vascular risk factors or CHD, so the distribution of these
factors is representative of the general nondiabetic population. In our analysis, we were then able to assess whether differences in vascular function were attributable to differences in risk factors between diabetic and nondiabetic groups or whether they were independent of classical risk factors and linked specifically to diabetes. We also examined the extent to which any defect associated with diabetes was restricted to those with poor recent glycemic control. Of the cohort of 400 subjects, 157 (39%) took part, 184 refused, and the remainder were ineligible (n=15) or not contactable (n=44). In total, 88 (56.1%) type 1 diabetic (54 men and 34 women) and 69 (43.9%) nondiabetic subjects (34 men and 35 women) participated. All were aged between 30 to 53 years. These subjects did not differ in risk factor profile, diabetes duration, control, or complications from the overall sample of 400.

Most female participants were studied in the follicular phase of the menstrual cycle, and this was the same for diabetic and nondiabetic women. None of the participants was undergoing nitrate therapy. Diabetic subjects who had hypoglycemia within 24 hours before their study were given a new appointment. All participants gave their informed consent. The study had the approval of the local ethics committee and was conducted over a period of 14 months.

### In Vivo Endothelial Function Test

Studies were performed between 1 to 2 hours after food and insulin therapy in a quiet temperature-controlled (24°C to 27°C) laboratory. Before the study, blood pressure in the right arm was measured 3 times using an automated digital monitor (Omrorn 705CP, OMRON Healthcare Europe B.V., the Netherlands) after 5 minutes of rest with the subject seated. Venous nonfasting blood was taken from the nondominant arm. A 27-gauge stainless steel needle (Cooper’s Needle Works) sealed to an epidural catheter (Portex) was inserted into the brachial artery of the nondominant arm under local anesthesia (1% lignocaine). Drugs were dissolved in 0.9% sodium chloride solution and were infused at 0.5 mL per minute using a constant infusion pump (Havard Apparatus). Forearm blood flow was recorded simultaneously in both arms by venous occlusion plethysmography as described previously.9 During measurements, upper arm collecting cuffs were inflated to 40 mm Hg for 10 seconds in each 15-second cycle, and circulation to the hands was excluded by inflating the wrist cuffs to 200 mm Hg. Flow was recorded after 25 minutes of rest following insertion of arterial cannula and was then measured in response to intrabrachial infusion of acetyicholine (ACh) (Sigma; doses of 25, 50, and 100 nmol/min, each dose for 3 minutes), bradykinin (BK) (Clinalfa; doses of 10, 30, and 100 pmol/min, each dose for 3 minutes), glyceryl trinitrate (GTN) (David Bull Laboratories; doses of 4, 8, and 16 nmol/min, each dose for 5 minutes), noradrenaline (Levophed; Sanofi Winthrop Ltd; doses of 4, 8, and 120 pmol/min, each dose for 5 minutes), and N5-monomethyl-L-arginine (L-NMMA) (Clinalfa; 1, 2, 4 μmol/min, each dose for 5 minutes). Each drug infusion was separated by a 10-minute saline washout period. The order of vasodilator infusions (ACh, BK, GTN) was randomized. Flow was recorded for approximately 10 seconds in every 15 seconds, and the mean of the last 4 measurements of each recording period was used for data analysis. Blood flow was expressed as milliliter of blood per 100 mL of forearm volume per minute (mL/100 mL per min). At the end of each study, a second blood sample was obtained and plasma was stored. Prestudy and poststudy plasma glucose was measured using the Integra method.10 The length and volume of the infused forearm arm were also measured.

### Statistical Analysis

The mean blood flow for each drug dose was assessed in each of the 4 diabetes sex groups (Figure, Table 2). From this model, the difference in response between diabetic and nondiabetic subjects is expressed as the percentage difference in absolute flow under drug adjusted for baseline flow, averaged across the 3 doses using the repeated-measures analysis of covariance model (XTREG procedure in STATA 6) with the data log transformed as appropriate.11 12 The relatively large sample size allowed exploration of the effects of concomitant risk factors by including these as covariates in the model. P<0.05 was considered significant.

### Results

#### Subject Characteristics

All subjects were white, except 1 diabetic woman and 1 nondiabetic woman who were Afro-Caribbean. Of the 34 diabetic women and 35 nondiabetic women, most (71% and 77%, respectively) were studied during the follicular phase of the menstrual cycle. Other background characteristics, medications, and prevalence of complications are shown in Table 1. No participants had a previous diagnosis of angina or myocardial infarction or any ischemic changes on resting ECG.

**Basal Forearm Blood Flow**

Basal flow (flow during saline infusion preceding the first drug infusion) was higher in men than in women (Table 1, P=0.001, adjusted for diabetes). Diabetic subjects had slightly higher basal flow than nondiabetic subjects (Table 1, P=0.1, adjusted for age and sex).

**Response to ACh, BK, and GTN**

The mean blood flow during saline and drug infusion is shown in Table 2. Acetylcholine, BK, and GTN produced a dose-dependent increase in flow in all 4 groups (Table 2 and Figure). There was a 2.73-fold increase in flow with ACh (averaged over the 3 doses) in nondiabetic subjects and a 2.11-fold increase in diabetic subjects. Adjusted for basal flow, age, and sex, the flow under ACh infusion was 18% (P=0.002) lower in the diabetic than nondiabetic group averaged across the 3 doses adjusted for basal flow (Table 3). Similarly, the diabetic group had a 17% lower response to GTN, but the BK response was similar between groups (6% lower in diabetic subjects; NS). The magnitude of the difference in response to ACh, BK, and GTN by diabetes was not strongly influenced by adjustment for basal flow (20%, 8%, and 18% lower response, respectively, in diabetic subjects before adjustment for basal flow).

**Effects of Sex on Responses**

The reduction in response to ACh associated with diabetes was greater in women (25% reduction, Table 3; P=0.01) than men (13% reduction), although this sex difference in diabetes-induced vascular dysfunction did not reach statistical significance (P=0.26 for diabetes by sex interaction). In nondiabetic subjects, ACh response was 23% greater in women than men (P=0.02), and this sex difference was less in diabetic subjects (11% difference, P=0.18), but again this did not reach significance (P=0.26 for the diabetes-sex interaction). There was no difference between men and women in the effect of diabetes on responses to other drugs.

**Effect of Risk Factors on the Diabetic Differences**

There was no association between prestudy or poststudy plasma glucose concentrations and any of the drug responses. Accordingly, adjusting for prestudy and poststudy plasma glucose concentrations did not alter the difference between
diabetic and nondiabetic subjects for any drug response. In all subjects combined, a higher HDL-C was significantly associated with a higher flow with ACh infusion (20% higher for every 1 mmol higher HDL-C, *P* = 0.0008). Higher triglycerides and body mass index (BMI) were associated with lower GTN response (3% lower for every 1 mmol/L of triglyceride, *P* = 0.018 and 2% lower for every kg/m² higher BMI, *P* = 0.02). Adjusting for these factors made little difference to the lower response to vasodilators in diabetic than nondiabetic subjects. Additional adjustment for other established risk factors did not alter this. On adjustment for HDL-C, LDL-C, triglycerides, pack years of smoking, alcohol consumption, systolic blood pressure, and BMI, diabetes continued to be associated with a lower response to ACh (22% and 20% lower, respectively). Age was not associated with blood flow response. Additional adjustment for forearm volume and flow in the contralateral arm during the experiment did not alter these conclusions.

Overall, 5 diabetic women, 8 diabetic men, 1 nondiabetic woman, and 1 nondiabetic man were taking antihypertensive drugs and 1 diabetic man was taking a statin. Twelve of the 13 diabetic subjects taking antihypertensive drugs were taking angiotensin-converting enzyme (ACE) inhibitors. No subjects were taking fibrates or diuretics. Among diabetic subjects, treatment with antihypertensive drugs was associated with a 15% higher response to BK (*P* = 0.04). Restricting the analysis to the 64 subjects (37 diabetic and 27 nondiabetic) who had never smoked, were normotensive, and were not taking any blood pressure or lipid-lowering drugs did not significantly alter the analysis for ACh or GTN (responses to ACh and GTN were 22% and 20% lower with diabetes, *P* = 0.02 and *P* = 0.001, respectively). However, for the BK response, restricting the analysis to those who were not taking antihypertensive drugs, the response was then significantly lower in diabetic subjects (9% lower, *P* = 0.04), and with additional restriction to those who had never smoked, were
Among diabetic subjects, a 1% increment in HbA1c was associated with a 7% reduction in ACh response (P=0.003) but was not related to responses to other vasodilators. The difference in ACh response between those with and without diabetes was 24% for diabetic subjects with HbA1c values above the median (8.6%) compared with 10% in those with values below the median. Interestingly, the higher HbA1c in diabetic women than men contributed to the attenuated sex difference in ACh response; on adjustment for HbA1c, the sex difference in ACh response increased to 18% (P=0.02), a value similar to that seen in nondiabetic subjects. Diabetes duration was not associated with vasodilator response, but the shortest diabetes duration was 7 years, so we were not able to assess the association of very short-term diabetes with drug responses. Only 14 diabetic subjects had microalbuminuria or macroalbuminuria, and response to vasodilators was not related to albuminuria; similar to the overall results, normoalbuminuric diabetic subjects had an ACh response that was 20% lower than the nondiabetic subjects. Neither self-reported history of retinopathy (present in 10 subjects) nor neuropathy was associated with drug response.
examined separately. Adjusting for prestudy and poststudy plasma glucose concentrations did not alter this result, and neither did adjusting for control arm flow. Because the infusion doses for L-NMMA and noradrenaline were deliberately chosen to give the same degree of vasoconstriction, we compared the responses to the drugs within the diabetic and nondiabetic groups (Table 3). There was no significant difference between diabetic and nondiabetic subjects in either sex with either vasoactive drugs. Between diabetic and nondiabetic groups (Table 3). There was no significant difference between diabetic and nondiabetic subjects in either sex with either vasoactive drugs. Between diabetic and nondiabetic subjects in either sex with either vasoactive drugs. Between diabetic and nondiabetic subjects in either sex with either vasoactive drugs. Between diabetic and nondiabetic subjects in either sex with either vasoactive drugs. Between diabetic and nondiabetic subjects in either sex with either vasoactive drugs.

### Table 3. Response to Drugs by Diabetes and Sex

<table>
<thead>
<tr>
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<th>Men</th>
<th>Women</th>
<th>Men and Women</th>
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<tr>
<td></td>
<td>Ratio of Flow During Drug Infusion to Flow at Baseline Averaged Across Doses*</td>
<td>% Difference (95%CI) in Flow Under Drug Adjusted for Age and Basal Flow (Diabetic vs Nondiabetic)</td>
<td>Ratio of Flow During Drug Infusion to Flow at Baseline Averaged Across Doses*</td>
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<tr>
<td></td>
<td>Diabetic</td>
<td>Nondiabetic</td>
<td>Diabetic</td>
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<tr>
<td>ACh</td>
<td>1.95</td>
<td>2.28</td>
<td>−13 (−25, 2)</td>
</tr>
<tr>
<td>BK</td>
<td>1.99</td>
<td>2.10</td>
<td>−3 (−13, 9)</td>
</tr>
<tr>
<td>GTN</td>
<td>1.93</td>
<td>2.35</td>
<td>−16 (−24, −7)§</td>
</tr>
<tr>
<td>NA</td>
<td>0.78</td>
<td>0.76</td>
<td>−3 (−17, 9)</td>
</tr>
<tr>
<td>L-NMMA</td>
<td>0.78</td>
<td>0.80</td>
<td>2 (−4, 9)</td>
</tr>
</tbody>
</table>

*The geometric mean of the ratio is shown because the data have a skewed distribution.

Blood flow response to drugs was defined as the percentage difference in absolute flow between diabetic and nondiabetic subjects, adjusted for baseline flow averaged across the 3 doses using the repeated-measures ANCOVA model.

†P<0.05; ‡P<0.01; §P<0.001.

### Discussion

This large study utilizing dose-response relationships to multiple drugs demonstrates that there is a substantial defect in response to endothelium-dependant dilators and NO donors in a general population of type 1 diabetic subjects. These findings are consistent with either reduced NO bioavailability or vascular smooth muscle responsiveness to NO. Our data strongly suggest that there is an additional specific defect in ACh-stimulated NO production in diabetes and that this is related to glycemic control. The loss of sex difference in CHD may be attributable in part to a greater impairment of endothelial function associated with elevated HbA1c in women.

The results of this study show that forearm resistance vessel responses to endothelium-dependent and endothelium-independent agonists are abnormal in individuals with type 1 diabetes. Forearm flow responses to ACh and GTN were significantly lower in diabetic men and women compared with a control group of healthy volunteers. Interestingly, the responses to BK were also diminished if subjects taking ACE inhibitors were removed from the analysis. This is expected, because ACE inhibitors enhance BK-induced vasodilatation and therefore would have artificially increased the overall BK response in the diabetic group as a whole. Perhaps surprisingly, the differences in vascular responses we detected were not accounted for by other conventional cardiovascular risk factors and were specific for diabetes. Indeed, it was those individuals with the highest HbA1c levels who showed the most marked impairment of ACh responses, suggesting that recent poor glycemic control is a determinant of endothelial function in type 1 diabetes. This has not been observed previously in studies of this type, but this is probably because the sample sizes of earlier studies have been smaller and subjects have been selected rather than drawn at random from a diabetic population (see Chan et al for review). Age was not associated with response, but this was not surprising given the narrow age range of participants.

Many animal models have indicated that endothelial function is impaired in diabetes, but results from studies in humans have been inconsistent. Some have shown defects in agonist-induced responses, others have shown defects in...
responses to NO donors, and a few have detected changes in the constriction seen in response to inhibition of basal NO synthesis.\textsuperscript{3,4} The present study shows diminished responses to agonists that stimulate endogenous NO release as well as to exogenous NO donors. Indeed, some of the decrease in response to the endothelium-dependent agonists could be accounted for by a decrease in responsiveness to GTN. This finding is consistent with the observation that in most of the studies to date, both in resistance vessels and conduit vessels, the response to an NO donor (GTN or sodium nitroprusside) was lower in type 1 diabetic subjects compared with controls, even if in some studies the difference did not reach statistical significance.\textsuperscript{6,9,15–17} The magnitude of the difference in GTN response we detected is such that a sample size of approximately 100 subjects would be required to have 90\% power to detect it. Our study of 157 subjects was sufficiently powered to detect this difference, whereas previous studies of forearm flow have not been. The simplest interpretation of our findings is that vascular responsiveness to NO is diminished in type 1 diabetes, either because of decreased target enzyme responses to NO or because NO is destroyed more rapidly by superoxide or some other reactant. In addition, there seems to be an additional specific effect on ACh responses, the mechanism of which is unclear at present.

The response to L-NMMA was not different between diabetic subjects and controls. This differs from previous findings (including our own) undertaken in small groups of selected diabetic subjects. Given the sample size and study design, it seems likely that the present findings more accurately reflect the situation in a general population of type 1 diabetic subjects. A normal response to L-NMMA in the presence of apparent decreased sensitivity to NO suggests that basal NO synthesis must be increased to maintain an equivalent level of basal NO-mediated dilatation in diabetic subjects and controls. Although we have not tested this directly, this interpretation would be consistent with the observation that overall NO synthesis is elevated rather than diminished in type 1 diabetes.\textsuperscript{18} It remains to be determined whether the increase in basal NO is a primary change that then downregulates target enzymes for NO or whether quenching of NO signaling leads to an increase in NO generation to restore basal NO-mediated dilatation to healthy levels.

Diminished endothelium-dependent responses or responses to NO donors has been demonstrated in the presence of virtually every risk factor for cardiovascular disease. Indeed, there is increasing evidence that impaired vascular responses in the forearm themselves are predictive of increased cardiovascular risk. In this context, it is important that this group of representative and relatively healthy type 1 diabetic subjects have significantly impaired vascular responses of a type associated with increased cardiovascular risk. It is now well recognized that diabetes abolishes the sex difference in cardiovascular risk, and one possibility is that differences in endothelial function account for this effect. Although we found that ACh responses were significantly higher in healthy women compared with men and that diabetes impaired the ACh responses to a greater extent in women than men, there was no statistically significant diabetes-sex interaction. From our results, we calculate that a sample size of 320 would be required to detect a significant sex difference in the effect of diabetes on vascular responses. Despite these considerations, it is nonetheless intriguing to note that a 1\% increase in HbA1c was associated with a 7\% reduction in ACh response. In this study, glycemic control was worse in women than men, and on adjustment for HbA1c, the sex difference in ACh responses was essentially the same between diabetic and nondiabetic subjects. Sex differences in glycemic control in adults with type 1 diabetes are not consistently found,\textsuperscript{19,20} but a greater deterioration of glycemic control in girls than boys at adolescence is well described.\textsuperscript{21} This raises the possibility that sex differences in glycemic control contribute to the apparently increased detrimental effects of type 1 diabetes on cardiovascular risk in women via effects on endothelial function. Clearly, additional studies would be required to test this hypothesis directly.

Like any study, this one has limitations. First, we studied diabetic subjects in their usual state and decided not to use a euglycemic clamp model. It is not known what difference such an approach may have made to the results, but we do know that plasma glucose concentrations before and after the study showed no relationship with drug responses, and adjusting for these covariates did not influence the results. Second, it may be argued that we should have matched our diabetic subjects and controls for the presence and levels of other risk factors. However, we deliberately took the approach of selecting a general population of diabetic subjects and a general population of nondiabetic controls of sufficiently large sample size to allow us to determine to what extent differences in the prevalence or severity of risk factors between the groups may account for changes in endothelial function. This approach is well established in trial design and prevents problems associated with selection bias of subjects and is more efficient than matching.\textsuperscript{22} Finally, measurements of albuminuria, lipids, and BMI were made 1 year before the vascular study, and it may be that contemporaneous measurements would have yielded a different result. However, these risk factors have high tracking, even over long periods of follow up (ie, the rank order of these values in a group of people is fairly constant) and would not be expected to change much in the course of a year.\textsuperscript{23}

The results of this study confirm that forearm resistance vessel function is abnormal in individuals with type 1 diabetes and that at least part of the defect is likely to be attributable to diminished responsiveness to NO. The effect is attributable to diabetes per se and is not attributable to clustering of other risk factors in this population; indeed, recent glycemic control is a major determinant of the responses to the endothelium-dependent agonist ACh. It would be interesting to determine whether the apparent decreased responsiveness to NO is also seen in other important cell types, such as platelets and white cells, because this may additionally predispose to atherogenesis.\textsuperscript{24} The small group of diabetic patients treated with ACE inhibitors and lipid-lowering drugs showed improved endothelial responses, and this is consistent with the concept that treating conventional risk factors is a priority in diabetic subjects, even if some of the endothelial dysfunction is mediated by other mechanisms.
It also indicates that interpretation of BK responses must be undertaken with caution if subjects are taking ACE inhibitors. It remains to be determined whether the loss of sex differences in cardiovascular risk in type 1 diabetes is a function of greater changes in NO pathways in women compared with men, but the present study raises the intriguing possibility that changes in glycemic control between diabetic men and women could contribute to greater vascular dysfunction in diabetic women.

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References

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