

Diabetic Retinopathy Is Related to Both Endothelium-Dependent and -Independent Responses of Skin Microvascular Flow

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OBJECTIVE—Endothelial dysfunction has been hypothesized as a possible pathogenic factor in the development of diabetic retinopathy (DR). We examined the relationship of DR to endothelium-dependent and endothelium-independent responses in skin microvascular flow.

RESEARCH DESIGN AND METHODS—Participants consisted of 224 individuals with diabetes: 85 with type 1 diabetes and 139 with type 2 diabetes. Sodium nitroprusside (SNP) and acetylcholine (ACh) were delivered across the skin by iontophoresis. Laser Doppler flowmetry was used to assess the skin microcirculation response to SNP (endothelium-independent response) and ACh (endothelium-dependent response). The presence and severity of DR were graded from retinal photographs using a standard protocol.

RESULTS—Of 224 participants, 64.3% had DR. After multivariable adjustment, participants with reduced responses to SNP or ACh were more likely to have DR, with an odds ratio (OR) of 2.33 (95% CI 1.09–5.01) for SNP and 2.20 (1.05–4.61) for ACh, comparing participants with responses below and above the median values. Participants with reduced responses (below the median) to both SNP and ACh were nearly four times more likely to have DR (OR 3.86 [1.45–10.3]) than those with SNP and ACh both above the median values.

CONCLUSIONS—The presence of DR was associated with a reduction in skin microcirculation responses to iontophoresis of both SNP and ACh, suggesting that vascular processes associated with both endothelial dysfunction and endothelial function-independent mechanisms may be pathogenically related to DR.

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Diabetes affects 285 million individuals worldwide (1), and diabetic retinopathy (DR) is the leading cause of blindness in the working-age population in most developed countries (2). Although it has been suggested that endothelial dysfunction may underlie the pathogenesis of DR (3), the few clinical and epidemiologic studies to date have found inconsistent associations of DR with indirect serum markers of endothelial

function (e.g., soluble vascular adhesion molecule-1) (4–9) or brachial flow-mediated dilation (FMD) (10–13), a more direct measurement of endothelial dysfunction (14).

Drug delivery to the skin by iontophoresis, accompanied by laser Doppler technology to measure microvascular flow, has been validated as a measure of endothelial function in the skin microcirculation (15). Responses of the skin

microcirculation to sodium nitroprusside (SNP) and acetylcholine (ACh) are measures of endothelium-independent and endothelium-dependent responses, respectively. Studies have shown a strong correlation between endothelial function assessed by FMD of the brachial artery and ACh increase in skin blood flow ($r = 0.92$, $P < 0.0001$) (16). Further study has shown that impaired skin microvascular response is associated with a range of cardiovascular risk factors and diseases (15).

In this study, we examined the relationship of DR with skin microvascular dysfunction as measured by iontophoresis and laser Doppler flowmetry in a clinical sample of patients with diabetes. Our aim was to establish whether DR was associated with systemic microvascular dysfunction evidenced in the skin and whether this was primarily driven by endothelial dysfunction.

RESEARCH DESIGN AND METHODS

We conducted a case-control study of DR among 224 individuals with diabetes, consisting of 85 with type 1 diabetes and 139 with type 2 diabetes. Participants were recruited from the diabetic eye clinics at the International Diabetes Institute, Melbourne, Australia, between October 2006 and April 2008. Detailed examination of the participants has been previously reported (3). In brief, individuals with known diabetes who were seen at the diabetic eye clinics and treated with oral hypoglycemic medications and/or insulin were eligible. Participants were excluded if aged older than 70 years, currently pregnant, had a previous mastectomy (with axillary clearance), previous vitreal surgery, had an arteriovenous fistula on the arm/forearm, a history of skin cancer on the arm/forearm, epilepsy, glaucoma, and/or had cataract on examinations for this study.

All participants underwent a standardized clinical examination, analysis of blood chemistry, retinal photographs, and assessment of skin microvascular dysfunction. Tenets of the Declaration of

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Helsinki were followed, institutional review board approval was granted, and written informed consent was obtained from all participants.

Skin microcirculation endothelial function

Skin microcirculation endothelial function was measured using a laser Doppler flowmetry technique that assessed the skin microcirculation responses to iontophoresis of SNP and ACh (15). Iontophoresis delivers the SNP and ACh vasodilators across the skin using a weak electrical current, with blood flow measured by laser Doppler flowmetry. Participants were seated comfortably in a room with an ambient temperature of 20–23°C. All tests were performed on both forearms.

A battery-powered current stimulator (WPI 502R) provided a direct current for drug iontophoresis. A laser Doppler flow meter (Moor Instruments, Axminster, U.K.) was used to measure the skin blood flux (in arbitrary perfusion units). Flux is a function of the volume of blood multiplied by velocity. Vascular responses to SNP (endothelial-independent vasodilator) and ACh (endothelial-dependent vasodilator) were recorded. SNP 1% and ACh 1% solutions in distilled water were used for iontophoresis. Doses of the vasoactive drugs were expressed as a total charge in millicoulombs (mC), determined by the product of current (0.2 mA) and stimulus duration (seconds). Doses of 16 mC (0.2 mA × 80 s) were used. “Mean baseline flux” was the mean flux measured over 120 s before iontophoresis, and “mean response flux” was the mean flux over 240 s after the iontophoresis. The responses to SNP or ACh were calculated as: [Mean response flux – Mean baseline flux]/[Mean baseline flux].

Assessment of DR

DR was graded from fundus photographs at the Centre for Eye Research Australia by graders masked to clinical details (3). A DR severity score was assigned for each eye using a modification of the Airlie House Classification system (17). Presence of DR was defined as any retinopathy signs in the right or left eye.

Assessment of other risk factors

A detailed questionnaire was used to obtain participant information, including medical history, current cigarette smoking, and the use of hypertension and dyslipidemia medications. Hypertension was defined as systolic blood pressure

(SBP) ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or current use of anti-hypertensive medications. Dyslipidemia was defined as cholesterol >5.5 mmol/L, or triglyceride >2.0 mmol/L, or current use of lipid-lowering medications. Height and weight were measured to determine BMI. Fasting blood samples were drawn from participants at suburban pathology centers for fasting blood glucose (FBG), cholesterol, and triglyceride levels within 2 weeks of the eye testing.

Statistical analysis

Mean responses to iontophoresis of SNP and ACh of the right and left arms were used. When only one was arm measured, responses in that arm were used. Responses were dichotomized by the median values into upper and lower half (50%) for analyses. Multiple logistic regression models were used to estimate odds ratios (ORs) and 95% CI for the presence of DR according to whether subjects were in the upper or lower half of the responses to iontophoresis. We adjusted for age and sex (model 1), and further adjusted for duration of diabetes, FBG level, SBP, fasting cholesterol and triglyceride levels, current smoking status, and use of anti-hypertensive and dyslipidemia medications (model 2). Responses to SNP and ACh were modeled separately initially, followed by further adjustment for ACh

response in models assessing SNP response, or SNP response in models assessing ACh response (model 3).

Finally, responses to SNP and ACh were assessed jointly by classifying four groups of responses, comprising group 1: those with responses in the upper half for both SNP and ACh (reference group), group 2: those with responses in the lower half for SNP but in the upper half for ACh, group 3: those with responses in the lower half for ACh but in the upper half for SNP, and group 4: those with responses in the lower half for both SNP and ACh. Analyses were performed in Stata 10.1 software (StataCorp, College Station, TX).

RESULTS—The participants were of white racial background. Selected characteristics of the 224 participants with diabetes, and of those with (n = 144) and without (n = 80) DR, are summarized in Table 1. Mean age was 56.5 ± 11.8 years, 59.4% were men, and 64.3% had DR. Those with DR had a longer duration of diabetes, a higher SBP, and were more likely to have hypertension. In addition, those with DR also had reduced responses to iontophoresis of SNP and ACh.

Individuals with reduced responses to iontophoresis of SNP or ACh were more likely to have DR (SNP: 70.3 vs. 50.5%; ACh: 71.0 vs. 50.5%; Fig. 1, Table 2). After

Table 1—Participant characteristics comparing subjects with and without DR

Variable	All subjects N = 224	DR present n = 144	DR absent n = 80	P*
Male sex	133 (59.4)	41 (51.3)	92 (63.9)	0.07
Smoking				
Current	16 (7.1)	14 (9.7)	2 (2.5)	0.11
Past	88 (39.3)	57 (39.6)	31 (38.8)	
Never	120 (53.6)	73 (50.7)	47 (58.8)	
Hypertension	137 (61.2)	99 (68.8)	38 (47.5)	0.002
Dyslipidemia	122 (54.5)	83 (57.6)	39 (48.8)	0.20
Age (years)	56.5 ± 11.8	57.4 ± 10.9	54.8 ± 13.3	0.11
Diabetes duration (years)	16.2 ± 10.5	18.6 ± 10.7	11.8 ± 8.67	<0.001
Blood pressure (mmHg)				
Systolic	128 ± 14.5	129.8 ± 14.9	124.8 ± 13.2	0.01
Diastolic	76.1 ± 8.9	75.9 ± 9.2	76.5 ± 8.4	0.64
BMI (kg/m ²)	30.4 ± 6.3	30.9 ± 6.4	29.8 ± 5.6	0.24
Hemoglobin A _{1c} (%)	7.9 ± 1.4	7.9 ± 1.1	7.8 ± 2.1	0.68
Glucose (mmol/L)	9.2 ± 3.7	9.3 ± 3.6	8.8 ± 4.0	0.50
Cholesterol (mmol/L)	4.6 ± 1.1	4.5 ± 1.0	4.6 ± 1.3	0.51
Triglyceride (mmol/L)	1.6 ± 1.1	1.7 ± 1.1	1.5 ± 0.9	0.44
SNP	5.0 ± 2.34	4.63 ± 2.10	5.58 ± 2.58	0.005
ACh	6.41 ± 3.15	5.92 ± 3.07	7.16 ± 3.13	0.007

Data are presented as n (%) or as mean ± SD. *Comparing those with and without DR in those with diabetes.

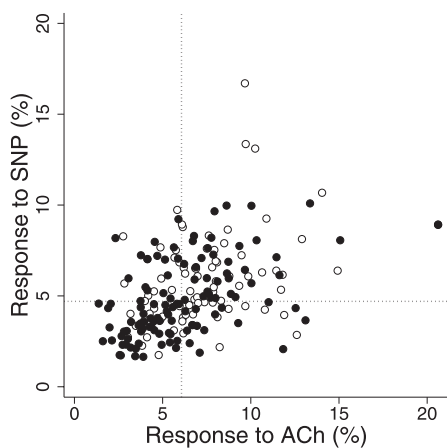


Figure 1—Scatterplots of the responses to SNP and ACh in people with diabetes. The black circle (●) indicates individuals with diabetic retinopathy.

adjusting for age, sex, duration of diabetes, FBG, SBP, fasting cholesterol and triglyceride levels, current smoking status, and use of antihypertensive and lipid-lowering medications, reduced responses to SNP or ACh were associated with increased odds of DR (SNP: OR 2.33 [95% CI 1.09–5.01], $P = 0.03$; ACh: 2.20 [1.05–4.61], $P = 0.04$, comparing lower vs. upper half of responses of each test, respectively). This was no longer statistically significant after further adjusting for ACh response in models

assessing SNP response, or vice versa (model 3).

Subjects with reduced responses to both SNP and ACh, compared with those with both responses above the median values, had higher odds of DR (OR 3.86 [95% CI 1.45–10.3], $P = 0.007$) after multivariable adjustment. Most of the diabetic individuals were those with responses in the upper half for both SNP and ACh (group 1) and in the lower half for both SNP and ACh (group 4).

A separate analysis by those with type 1 and type 2 diabetes showed the association of DR and reduced iontophoretic responses remained significant only in those with type 2 diabetes, with the SNP responses (upper vs. lower half), and those with reduced responses to both SNP and ACh (Supplementary Table A1). There were only 85 individuals with type 1 and 139 with type 2 diabetes.

CONCLUSIONS—This study demonstrated that among patients with diabetes, those with DR had a reduction in the skin microvascular responses to iontophoresis of both SNP (endothelium-independent response) and ACh (endothelium-dependent response). Patients with a reduction in responses to SNP or ACh were two times more likely to have DR, whereas those with a reduction in responses to both SNP and ACh were four times more likely to have DR. These associations were independent

of major risk factors for diabetes and cardiovascular diseases, including duration of diabetes, glycemia, and blood pressure. Our findings suggest that DR is closely linked with systemic vascular disease processes, as evidenced in the skin, that reflect a combination of endothelium-dependant dysfunction and endothelium-independent mechanisms.

To our best knowledge, this is the first study to examine DR and changes in skin microcirculation, as measured by laser Doppler flowmetry in response to iontophoresis of SNP and ACh. ACh is known to stimulate nitric oxide (NO) production in endothelial cells (endothelium-dependent vasodilation) (18,19), whereas SNP is an NO donor to vascular smooth muscle cells (endothelium-independent vasodilation). Theoretically, a reduction in vascular response in ACh alone, with no concurrent reduction in SNP response, would be indicative of endothelial dysfunction. Most of the diabetic individuals in our study were those with responses for both SNP and ACh in the upper half (group 1) and in the lower half (group 4). Owing to the relatively small sample sizes, we were unable to adequately compare those with high SNP and high ACh responses versus those with high SNP but low ACh responses (i.e., a comparison of endothelial function). In addition, our study could not discriminate whether abnormal responses were due to decreased

Table 2—Association between skin iontophoretic responses to SNP and ACh with DR

Groups	Range	N	DR	Model 1*	P	Model 2†	P	Model 3‡	P
			n (%)	OR (95% CI)		OR (95% CI)		OR (95% CI)	
Response to									
SNP (endothelium-independent)									
Upper half	4.70–17.2	95	48 (50.5)	1.0	—	1.0	—	1.0	—
Lower half	1.46–4.67	101	71 (70.3)	2.20 (1.19–4.07)	0.01	2.33 (1.09–5.01)	0.03	2.14 (0.96–4.77)	0.06
ACh (endothelium-dependent)									
Upper half	6.08–20.5	97	49 (50.5)	1.0	—	1.0	—	1.0	—
Lower half	1.26–6.06	99	71 (71.0)	2.25 (1.23–4.12)	0.008	2.20 (1.05–4.61)	0.04	1.98 (0.91–4.28)	0.08
Both SNP and ACh§									
Upper half of both SNP and ACh	—	68	34 (50)	1.0	—	1.0	—	—	—
Lower half of SNP, upper half of ACh	—	29	15 (51.7)	1.01 (0.41–2.49)	0.98	0.84 (0.28–2.46)	0.74	—	—
Upper half of SNP, lower half of ACh	—	27	14 (51.9)	1.01 (0.41–2.46)	0.99	0.86 (0.28–2.63)	0.79	—	—
Lower half of both SNP and ACh	—	72	56 (77.8)	3.30 (1.54–7.10)	0.002	3.86 (1.45–10.3)	0.007	—	—

Boldface type indicates significant interaction between responses to SNP and ACh (P for interaction term < 0.05). *Model 1: Adjusted for age and sex. †Model 2: Adjusted for covariates in model 1 plus diabetes duration, FBG, total cholesterol and triglyceride levels, BMI, SBP, current smoking status, and use of antihypertensive and lipid-lowering medications. ‡Model 3: Adjusted for covariates in model 2 plus ACh response with SNP response, or SNP response with ACh response. §Participants divided into four groups according to response to SNP and ACh (see text).

production of NO, increased destruction or inactivation of NO, or decreased vascular smooth muscle cell responsiveness to it.

Nevertheless, our finding of an association of DR with a reduction in skin responses to both ACh and SNP implies a likely more complex mechanism in DR pathogenesis than pure endothelial dysfunction alone. This may explain the inconsistent findings of DR and systemic measures of endothelial dysfunction such as serum markers (e.g., soluble vascular adhesion molecule-1) (4–9), and brachial FMD (10–13).

Our observation that DR is associated with a combined reduction in responses to endothelial-dependent and independent vasodilatation may also reflect microvascular sclerosis (i.e., a more physical loss of vasodilator reserve) (19,20). However, the overall vascular dysfunction probably involves a complex interaction between functional and structural abnormalities in the microcirculation (21,22). We have previously shown that DR may be associated with retinal endothelial dysfunction in the same cohort (3) and that this may be due to differences in the vascular beds. However, our finding of DR with abnormal systemic microcirculation may provide some insights into the relationships between DR and increased risks of cardiovascular diseases, including new measures of subclinical cardiovascular disease such as coronary artery calcification and cardiac remodeling (23).

The strengths of this study include the photographic assessment of DR using standardized grading protocols, and one person (T.T.N.), masked to DR status, performed and supervised the assessment of the skin microcirculation responses to SNP and ACh using laser Doppler flowmetry.

Limitations of this study should also be noted. First, the cross-sectional nature provides no temporal information on the associations reported. Second, our findings are only applicable to individuals with diabetes, not pregnant, and aged 70 years or younger. Third, the analysis of the subgroups of level of DR was not feasible due to the small number in each group, and thus, our study was not sufficiently powered to address this. In addition, our study was not sufficiently powered to analyze the relationships in type 1 and type 2 diabetes separately, with only 85 with type 1 and 139 with type 2 diabetes. These are possible future study questions.

Limitation of the method used to assess endothelial function includes the use of single-point laser Doppler flowmetry to assess responses to iontophoresis of SNP and ACh. Laser Doppler flowmetry is less reliable and reproducible than laser Doppler imaging systems, which measure perfusion over a larger area and produce a detailed perfusion map (15). In addition, comparison with future studies would be difficult owing to differences in apparatus, iontophoresis protocols, and environmental conditions. A standardization of techniques would help to resolve this.

In conclusion, we demonstrated an association of DR with reduced responses to iontophoresis of both SNP and ACh in the skin microcirculation. Our findings suggest associations of DR with systemic microangiopathy reflect a combination of endothelial dysfunction and endothelial function-independent mechanisms. These findings provide further insights into possible mechanisms of systemic risk factors on development of DR.

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T.T.N. researched data and wrote the manuscript. J.E.S. set up and supervised the project, contributed to discussion, and reviewed and edited the manuscript. C.R. researched data and reviewed and edited the manuscript. R.K. and J.J.W. analyzed statistics and reviewed and edited the manuscript. A.J.K. researched data and reviewed and edited the manuscript. T.Y.W. set up and supervised the project, contributed to discussion, and reviewed and edited the manuscript.

References

- Shaw JE, Sicree RA, Zimmet PZ. Global estimates of the prevalence of diabetes for 2010 and 2030. *Diabetes Res Clin Pract* 2010;87:4–14
- Cheung N, Mitchell P, Wong TY. Diabetic retinopathy. *Lancet* 2010;376:124–136
- Nguyen TT, Kawasaki R, Wang JJ, et al. Flicker light-induced retinal vasodilation in diabetes and diabetic retinopathy. *Diabetes Care* 2009;32:2075–2080
- van Hecke MV, Dekker JM, Nijpels G, et al. Inflammation and endothelial dysfunction are associated with retinopathy:

the Hoorn Study. *Diabetologia* 2005;48:1300–1306

- Matsumoto K, Sera Y, Ueki Y, Inukai G, Niuro E, Miyake S. Comparison of serum concentrations of soluble adhesion molecules in diabetic microangiopathy and macroangiopathy. *Diabet Med* 2002;19:822–826
- Siemianowicz K, Francuz T, Gminski J, Telega A, Syzdól M. Endothelium dysfunction markers in patients with diabetic retinopathy. *Int J Mol Med* 2005;15:459–462
- Spijkerman AM, Gall MA, Tarnow L, et al. Endothelial dysfunction and low-grade inflammation and the progression of retinopathy in type 2 diabetes. *Diabet Med* 2007;24:969–976
- Boulbou MS, Koukoulis GN, Petinaki EA, Germenis A, Gourgoulis KI. Soluble adhesion molecules are not involved in the development of retinopathy in type 2 diabetic patients. *Acta Diabetol* 2004;41:118–122
- Koga M, Otsuki M, Kubo M, Hashimoto J, Kasayama S. Relationship between circulating vascular cell adhesion molecule-1 and microvascular complications in type 2 diabetes mellitus. *Diabet Med* 1998;15:661–667
- Malecki MT, Osmenda G, Walus-Miarka M, et al. Retinopathy in type 2 diabetes mellitus is associated with increased intima-media thickness and endothelial dysfunction. *Eur J Clin Invest* 2008;38:925–930
- Suetsugu M, Takebayashi K, Aso Y. Association between diabetic microangiopathy and vascular endothelial function evaluated by flow-mediated vasodilatation in patients with type 2 diabetes. *Int J Clin Pract* 2007;61:920–926
- van Hecke MV, Dekker JM, Nijpels G, et al. Are retinal microvascular abnormalities associated with large artery endothelial dysfunction and intima-media thickness? The Hoorn Study. *Clin Sci (Lond)* 2006;110:597–604
- Nair BM, Viswanathan V, Snehalatha C, Mohan RS, Ramachandran A. Flow mediated dilatation and carotid intimal media thickness in South Indian type 2 diabetic subjects. *Diabetes Res Clin Pract* 2004;65:13–19
- Celermajer DS, Sorensen KE, Gooch VM, et al. Non-invasive detection of endothelial dysfunction in children and adults at risk of atherosclerosis. *Lancet* 1992;340:1111–1115
- Turner J, Belch JJ, Khan F. Current concepts in assessment of microvascular endothelial function using laser Doppler imaging and iontophoresis. *Trends Cardiovasc Med* 2008;18:109–116
- Debbabi H, Bonnin P, Ducluzeau PH, Lefthérotis G, Levy BI. Noninvasive assessment of endothelial function in the skin microcirculation. *Am J Hypertens* 2010;23:541–546

17. Diabetic Retinopathy Study Research Group. Design, methods, and baseline results. A modification of the Airlie House classification of diabetic retinopathy (DRS Report #7). Prepared by the Diabetic Retinopathy. *Invest Ophthalmol Vis Sci* 1981; 21:1b-226b
18. Caballero AE, Arora S, Saouaf R, et al. Microvascular and macrovascular reactivity is reduced in subjects at risk for type 2 diabetes. *Diabetes* 1999;48:1856-1862
19. Ramsay JE, Simms RJ, Ferrell WR, et al. Enhancement of endothelial function by pregnancy: inadequate response in women with type 1 diabetes. *Diabetes Care* 2003; 26:475-479
20. Tooke JE. Microvascular function in human diabetes. A physiological perspective. *Diabetes* 1995;44:721-726
21. Khan F, Elhadd TA, Greene SA, Belch JJ. Impaired skin microvascular function in children, adolescents, and young adults with type 1 diabetes. *Diabetes Care* 2000; 23:215-220
22. Khan F, Green FC, Forsyth JS, Greene SA, Morris AD, Belch JJ. Impaired microvascular function in normal children: effects of adiposity and poor glucose handling. *J Physiol* 2003;551:705-711
23. Cheung N, Wong TY. Diabetic retinopathy and systemic vascular complications. *Prog Retin Eye Res* 2008;27: 161-176