Relationship between changes in brachial artery flow-mediated dilation and basal release of nitric oxide in subjects with Type 2 diabetes

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Nitric oxide (NO) bioavailability is thought to play an important atheroprotective role because it inhibits numerous pro-atherosclerotic processes implicated in the progression of atherosclerosis, including LDL oxidation, platelet aggregation, monocyte adhesion, and smooth muscle cell proliferation (4, 13, 30, 35). The vascular endothelium releases NO basally (33) and in combination with other vasodilators (e.g., prostacyclin and endothelium-derived hyperpolarizing factor) in response to increases in flow-associated shear stress (25).

Anderson et al. (1) were the first to demonstrate that the latter response in a conduit artery results in dilation that could be observed noninvasively via ultrasound imaging (flow-mediated dilation; FMD), whereas Sinoway et al. (32) also provided early evidence for flow-mediated conduit artery dilation. The FMD approach involves a short period of occlusion of the forearm that evokes forearm resistance vessel dilation (11). On release of occlusion, there is a substantial but transient elevation in brachial artery flow velocity providing an increased shear stress stimulus and resulting in FMD.

In the early 1990s, Celermajer et al. (5–8) hypothesized and demonstrated that endothelial vasodilator dysfunction could be detected between populations of clinical interest by examining this FMD response. Subsequent studies (18, 21, 24) appeared to support the hypothesis that this FMD was largely mediated by NO. Since then, measurement of brachial artery diameter change after a brief period of forearm ischemia, the so-called FMD technique, has become a common in vivo NO-vasodilator system bioassay (14), due largely to its noninvasive nature.

With the caveat that edge-detection and wall-tracking software are employed, the measured response appears relatively operator independent and reproducible (36).

However, an important assumption is made when FMD results are used as a surrogate marker of endothelial dysfunction and early atherogenesis (34); that endothelial NO function stimulated by a transient increase in shear stress relates to long-term basal NO-mediated vasoprotection, the latter being of more potential importance given that it is the most prevalent form of NO bioavailability in any 24-h period.

Although some work has been done to determine whether stimulated endothelial vasodilator function is similar in peripheral versus coronary arteries (2) or between shear-stimulated conduit and acetylcholine-stimulated resistance vessels (12, 16), the critical link between FMD and basal endothelial NO function has not been investigated. We have previously reported vascular function data in subjects with Type 2 diabetes who underwent randomized crossover studies of the effect of either exercise training (23) or angiotensin II type 1 receptor blockade (9, 10). However, no comparisons between stimulated and basal NO-mediated vascular function were included in these studies. We have therefore used these data sets to perform a pooled analysis of within-subjects comparisons between brachial artery FMD responses and forearm blood flow (FBF) responses to N0-monomethyl-L-arginine (l-NMMA). This analysis tested the following hypotheses: 1) conduit artery-stimulated endothelial NO function...
(FMD) reflects basal resistance vessel endothelial NO function before interventions known to improve endothelial function and 2) intervention-induced improvements in FMD reflect improvements in basal resistance vessel endothelial NO function.

METHODS

Subjects

Subjects with Type 2 diabetes without evidence of microvascular or macrovascular complications underwent a screening program consisting of a medical history and examination, hematological and biochemical profile, including measurement of blood glucose, glycated hemoglobin, serum electrolytes, urea and creatinine, uric acid, liver function, and serum lipids. The following were excluded: smokers and those with renal impairment or proteinuria, hepatic impairment, gout or hyperuricemia, more than mild hypercholesterolemia (total cholesterol > 6.5 mmol/l) or hypertension (systolic blood pressure > 160 mmHg). Eleven patients were taking metformin and, additionally, seven were taking glibenclamide and two metformin plus glipizide. Medications remained unchanged during the study. None had significant microalbuminuria or retinopathy. The mean glycated hemoglobin at entry was 8.0 ± 0.5% (normal range, 4.3–6.0%), indicating moderate to good glycemic control. The study protocol was approved by Royal Perth Hospital Ethics Committee, and subjects gave written, informed consent.

Study Design

After preliminary screening and baseline assessments, subjects in the exercise training study (n = 9) were randomly assigned to remain sedentary or to perform exercise training for 8-wk periods, followed by crossover. The exercise training protocol and assessment procedures are outlined below. Subjects were requested to make no changes to their diet, therapy, or other routines for the duration of the study. Interventions commenced within 7 days of the completion of baseline assessments, and all repeat assessments, including resistance and conduit vessel function, were performed within 7 days of the cessation of exercise training or control periods. Green’s laboratory (23) has previously published vascular function data on the effect of exercise training in these subjects with Type 2 diabetes, although this study did not include 1-NMMA data or analysis of the relationship between FMD and 1-NMMA responses.

Subjects in the angiotensin blockade group (n = 8) undertook a similarly designed crossover protocol involving 4 wk of losartan (50 mg, daily; Merck, Sharp & Dohme) or a similarly packaged placebo. The validity of the active drug/placebo randomization was checked by tablet analysis using an HPLC qualitative method. At each visit, the biochemical and hematological parameters were repeated. There were no adverse side effects. Although we have previously published data on the effect of losartan on conduit (10) and resistance (9) vessel function in subjects with Type 2 diabetes, no data on the relationships between FMD and 1-NMMA responses were included in these studies.

Assessment of Vascular Endothelial Function

Vascular endothelial function assessments were conducted in a quiet, temperature-controlled environment, at separate attendances for conduit and resistance vessel function. Repeat investigations were performed at the same time of day for individual subjects. Subjects fasted for 8 h, abstained from alcohol and caffeine for 12 h, and did not perform any exercise for 24 h before assessments.

Assessment of conduit vessel stimulated endothelial NO function. Resting supine, the nondominant arm was extended and immobilized with foam supports and positioned at an angle of ~80° from the torso. Heart rate (HR) was continuously monitored with a three-lead ECG, and mean arterial pressure was determined from an automated sphygmomanometer (Dinamap 8100, Critikon) on the contralateral arm. Resting HR and blood pressure measures were recorded after 30 min of supine rest in all subjects.

A rapid inflation/deflation pneumatic cuff was then positioned on the imaged arm immediately distal to the olecranon process to provide a stimulus to forearm ischemia (11). An 11-MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (Aspen, Acuson) was used to image the brachial artery in the distal third of the upper arm. When an optimal image was attained, the probe was held stable in a stereostatic clamp. Ultrasound parameters were set to optimize longitudinal, B-mode images of the lumen/arterial wall interface. After 20-min rest, baseline images were recorded on a S-VHS video cassette recorder (SVO-9500 MDP, Sony, Tokyo, Japan) over 2 min. The forearm cuff was then inflated to 200 mmHg for 5 min. Images were recorded 30 s before cuff deflation and for 2 min after deflation. After 15-min rest, to allow arterial diameter to return to baseline, another 2-min baseline recording was made before administration of a sublingual 400-μg spray dose of glyceryl trinitrate (GTN) with images recorded for a further 5 min.

Brachial artery diameters were analyzed by using custom-designed edge-detection and wall-tracking software that minimizes investigator bias and has the power to detect an absolute change in FMD of 2% in a crossover design study with only six subjects (36). Briefly, an edge-detection algorithm averages >300-diameter measurements per frame, with 20–30 frames/s assessed. Those average diameter measures that coincide with the ECG R wave (also autodetected), i.e., occurring at end-diastole, were subsequently analyzed using a third-order polynomial curve (36). FMD responses were then calculated from the peak value derived from this polynomial curve, related to the average of all R wave-gated diameters collected during the baseline period preceding either the FMD or GTN manipulations. The mean intraobserver coefficient of variation of repeated measures of FMD with the use of this software is 6.7%, which is significantly lower than that for traditional manual methods (36).

Assessment of basal endothelial NO vasodilator function. While subjects lay in the supine position, a 20-gauge cannula (arrow) was inserted into the brachial artery of the nondominant arm, under local anesthesia with <2 ml of 1% lignocaine, to infuse vasoactive agents and sterile saline and for blood sampling and measurement of intraarterial pressure. Subjects were then positioned with elbows at heart level and hands at a comfortable height to allow forearm venous drainage. Pneumatic cuffs (SC10 and SCS, Hokanson) and strain gauges (SG 24, Medasonics; Fremont, CA) were positioned for FBF measurements. Wrist and upper arm cuffs were connected to rapid inflation devices (E-20 and AG 101, Hokanson); strain gauges were positioned 8–10 cm distal to the olecranon process of each arm. Strain-gauge placement and hand and elbow elevation were the same for repeat tests. An online microcomputer (SPG 16, Medasonics) sampled amplified output from the strain gauges at 75 Hz, which was displayed in real time. A software program controlled cuff inflation/deflation as well as data acquisition, storage, and display to ensure that blood flow measurements were synchronized with upper arm cuff inflation. Arterial pressure was monitored continuously with a Hewlett-Packard 78353A monitoring system. After a 20-min stabilization period subsequent to cannulation, ACh (Miochol; Ciba Vision) was infused at 10, 20, and 40 μg/min, each for 3 min. A further 20-min period of saline infusion ensued, followed by sodium nitroprusside (SNP) infusion (David Bull) at 2, 4, and 8 μg/min, each for 3 min. Finally, after another washout period, 1-NMMA (CA-11, Clinalfa, Switzerland) was infused at 2, 4, and 8 μmol/min, each for 5 min. All solutions were prepared aseptically immediately before infusion.
Exercise Training Protocol

Those subjects who underwent exercise training attended three supervised exercise training sessions per week. Circuit training sessions were performed at the Cardiac Gymnasium, Royal Perth Hospital, with the focus on the large muscles of the lower limbs. They were also instructed on correct lifting techniques to avoid the Valsalva maneuver. The 8-wk circuit training protocol involved a combination of resistance training, cycle ergometry, and treadmill walking. The resistance exercises were alternated with cycle stations at a work-to-rest ratio of 45:1 s. Subjects performed one lift every 3 s, completing 15 lifts in the 45-s work period. At completion of the circuit, subjects performed an additional 5 min of treadmill walking. Training intensity and duration were progressively increased during the first 2 to 3 wk, as tolerated. Resistance intensity commenced at 55% of pretraining one-repetition maximum and increased to 65% at week 6. Cycling and treadmill walking intensities were initially 70% of HRpeak, determined to 85% of HRpeak at the end of week 1. After the interventions, baseline brachial artery diameter preceding these interventions was 4.21 ± 0.13 mm, and percent FMD was 3.81 ± 0.72% (Fig. 1A).

FMD was not significantly correlated with the FBF response to L-NMMA, expressed as area under the dose-response curve (r = −0.05; P = 0.85, Fig. 2A) or as the effect of the maximum dose (8 μmol/min) of L-NMMA (r = −0.10; P = 0.69). Similarly, there were no significant correlations within each of the subgroups between FMD and L-NMMA responses (exercise training FMD vs. L-NMMA, r = −0.14, P = 0.72; and losartan FMD vs. L-NMMA, r = 0.45, P = 0.26). ACh area under the dose-response curve responses were also not significantly correlated with those to L-NMMA (r = 0.29; P = 0.24), and the responses to maximal doses of ACh and L-NMMA were not significantly related (r = 0.18; P = 0.48). O’Driscoll’s laboratory (16) has previously reported that ACh and FMD responses are not significantly correlated, and this was also true in the present study (r = 0.07, P = 0.79 for peak ACh response; r = −0.06, P = 0.82 for ACh AUC). A significant correlation was evident between entry measures of SNP and GTN (r = 0.60, P = 0.01).

Do Changes in FMD After Intervention Reflect Changes in Basal NO Dilator Function?

After the interventions, baseline brachial artery diameter was 4.15 ± 0.15 mm (P = 0.37 vs. preintervention) and FMD 6.15 ± 0.13% (P < 0.0005 vs. preintervention), respectively. The FMD responses to both exercise training (P < 0.001) and losartan administration (P < 0.05) significantly increased after these interventions. Responses to GTN administration did not differ before and after the interventions (15.21 ± 1.38 vs. 13.65 ± 1.43%, P = 0.95). FBF ratio responses to ACh significantly increased with the interventions (Table 2), and a significant main effect for intervention was evident by two-way ANOVA (P < 0.05). FBF ratio responses to SNP also increased (dose 2; see Table 2), although no significant effect was evident by two-way ANOVA. No differences were evident in L-NMMA responses at any dose or by ANOVA (P = 0.7, Fig. 1B).

Correlations between pooled changes in FMD and L-NMMA are depicted in Fig. 2B. There were no statistically significant

Table 1. Baseline characteristics of patients with Type 2 diabetes

<table>
<thead>
<tr>
<th></th>
<th>Exercise Training Group</th>
<th>Losartan Administration Group</th>
<th>Combined Group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Total Chol, mmol/l</td>
<td>4.8±0.4</td>
<td>4.7±0.3</td>
<td>4.5±0.2</td>
</tr>
<tr>
<td>LDL Chol, mmol/l</td>
<td>2.6±0.3</td>
<td>2.7±0.3</td>
<td>2.8±0.2</td>
</tr>
<tr>
<td>HDL Chol, mmol/l</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Blood glucose, mmol/l</td>
<td>11.2±1.2</td>
<td>10.3±0.9</td>
<td>7.6±1.7</td>
</tr>
<tr>
<td>Glycated hemoglobin, %</td>
<td>8.2±0.5</td>
<td>7.9±0.4</td>
<td>7.7±0.9</td>
</tr>
<tr>
<td>Rest heart rate, beats/min</td>
<td>70±3</td>
<td>69±3</td>
<td>67±3</td>
</tr>
<tr>
<td>VO2peak, ml/kg·min⁻¹</td>
<td>22.6±1.7</td>
<td>25.2±1.8†</td>
<td>22.6±1.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. Pre and Post, pre- and postinterventions, respectively; Chol, cholesterol; VO2peak, Peak O2 consumption (derived from exercise group only). *P < 0.05; †P < 0.01.
exercise training or angiotensin II type 1 receptor blockade. Despite this, no correlation existed between FMD and FBF responses to l-NMMA infusion either at entry to the studies or in response to the interventions administered. These data indicate that conduit artery-stimulated, NO-mediated vasodilator function and changes in response to intervention do not reflect basal resistance vessel NO function.

The reactive hyperemia-induced FMD approach, introduced by Celermajer and colleagues (8) and recently described by an international task force (11), has become a popular method of assessing function of the NO-dilator system in vivo. Although the FMD approach, when correctly applied (14, 28), is thought to represent NO-mediated endothelial function (18, 24), it is only recently that the physiology underlying the response has been investigated (27). Hence, despite the technique being related to prognosis and outcome in groups with cardiovascular disease (15, 35), important questions remain regarding the purported use of this measure as a barometer of cardiovascular disease risk (14, 28, 34). Although NO-mediated dilation of peripheral conduit arteries correlates with that in the coronary vessels (2), likely due to the fact that atherosclerosis is a systemic disease process, it is less clear whether stimulated NO production, as occurs in response to the shear stress associated with the 5-min ischemic stimulus used for FMD assessment, relates to basal NO function. Vallance and colleagues (33) were the first to establish that NO is produced both basally and in response to agonist stimulation in vivo, whereas earlier animal studies suggested that the physiological stimulus to NO production involved increased flow or shear stress (26, 31). More recently, distinct signal transduction pathways associated with stimulated and basal NO production and bioavailability have been elucidated (37). Because it seems reasonable to infer that clinical benefit in terms of vascular health might be associated, perhaps predominantly, with improvements in basal rather than stimulated NO-dilator system function, we were interested in the extent to which the FMD approach, a contrived stimulus to NO function, relates to basal NO bioavailability. To this end we compared FMD and l-NMMA responses in subjects with Type 2 diabetes who undertook randomized crossover trials of either exercise training or losartan administration. Our results indicate that stimulated NO-mediated vasodilation in conduit arteries does not correlate with basal NO-related resistance vessel endothelial function in this population.

Several explanations exist for the lack of association between FMD and l-NMMA responses we observed. As mentioned above, shear stress-mediated NO-dependent vasodilation relies on signal transduction pathways that are, to some extent, distinct from those associated with basal NO production, and these pathways may be differentially affected by the interventions that we administered. Alternatively, or perhaps additionally, it is possible that the differences in the techniques used or vessel beds studied contributed to the lack of correlation. A small number of previous studies have compared strain-gauge and ultrasound-derived measures of resistance and conduit vessel function in humans. Irace et al. (17) reported a significant correlation between FMD and ACh dose-response curves in a study of 10 healthy subjects and 6 patients with obesity or hypertension, whereas Lind et al. (22) and Eskurza et al. (12) found no correlation between brachial FMD and the FBF responses to muscarinic agonists. Finally, we recently

DISCUSSION

To our knowledge, this is the first study to compare conduit artery FMD responses to basal NO-mediated FBF measurements derived from the same subjects pre- and postintervention. We report data from subjects with Type 2 diabetes who exhibited significant improvements in FMD in response to...
reported no association between FMD and ACh responses in 43 subjects with diverse cardiovascular risk factors who undertook exercise training (16). These studies suggest that the mechanisms responsible for adaptations in the vasculature may differ according to the vascular territory involved or, possibly, that assessments using strain-gauge plethysmography differ to those using high-resolution ultrasound. In the present study we also assessed the relationship between measures of stimulated and basal NO-mediated function within the same vessel bed and using the same technical approach; resistance vessel plethysmographic responses to ACh significantly increased, whereas no changes were evident in L-NMMA responses and no correlation was present between the magnitude of changes in these variables and basal NO-mediated function within the same vessel bed either exercise or losartan groups. These data indicate that no relationship exists between stimulated conduit and basal resistance vessel, endothelium-dependent NO-dilator system function in vivo in subjects with Type 2 diabetes.

Table 2. Absolute blood flows in infused and noninfused forearms, before and after exercise training or losartan interventions

<table>
<thead>
<tr>
<th></th>
<th>Infused Arm Flows</th>
<th>Noninfused Arm Flows</th>
<th>%Change in FBF Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
</tr>
<tr>
<td>Resting FBF</td>
<td>2.18±0.18</td>
<td>2.26±0.25</td>
<td>2.18±0.12</td>
</tr>
<tr>
<td>ACh infusion</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>10 µg/min</td>
<td>4.12±0.82</td>
<td>5.23±1.07</td>
<td>2.38±0.18</td>
</tr>
<tr>
<td>20 µg/min</td>
<td>5.41±0.80</td>
<td>8.08±1.24</td>
<td>2.26±0.17</td>
</tr>
<tr>
<td>40 µg/min</td>
<td>9.43±1.25</td>
<td>11.85±1.60</td>
<td>2.35±0.18</td>
</tr>
<tr>
<td>Pre-SNP baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SNP infusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µg/min</td>
<td>7.44±0.51</td>
<td>7.77±0.75</td>
<td>2.34±0.21</td>
</tr>
<tr>
<td>4 µg/min</td>
<td>9.57±0.81</td>
<td>10.26±0.97</td>
<td>2.26±0.23</td>
</tr>
<tr>
<td>8 µg/min</td>
<td>13.01±0.98</td>
<td>13.69±1.32</td>
<td>2.23±0.17</td>
</tr>
<tr>
<td>Pre-L-NMMA baseline</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>L-NMMA infusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 µmol/min</td>
<td>1.74±0.21</td>
<td>1.87±0.20</td>
<td>2.13±0.18</td>
</tr>
<tr>
<td>4 µmol/min</td>
<td>1.64±0.18</td>
<td>1.61±0.16</td>
<td>2.17±0.17</td>
</tr>
<tr>
<td>8 µmol/min</td>
<td>1.52±0.17</td>
<td>1.52±0.17</td>
<td>2.19±0.18</td>
</tr>
</tbody>
</table>

Values are means ± SE (in ml forearm⁻¹ min⁻¹). FBF, forearm blood flow. P values relate to differences in ratios, pre- vs. postinterventions (paired t-test). Two-way ANOVA revealed significant main effect of intervention for ACh responses (P < 0.05); no main or interaction effect was evident for N⁵-monomethyl-L-arginine (L-NMMA) or sodium nitroprusside (SNP) responses.
stimulated NO-mediated responses can be differentially regulated within the same vessel bed. These data and our previous findings therefore concur with other evidence, reviewed elsewhere (19), which suggests that distinct changes in vascular function can occur according to the vascular territory studied, the type of vascular function assessed (stimulated vs. basal), or the mechanisms involved in the evoked responses.

There are several potential limitations of the present study. The sample was limited to subjects with Type 2 diabetes, and we cannot rule out the possibility of different relationships existing between FMD and L-NMMA responses in healthy subjects or those with more impaired vascular function. It is also conceivable that a longer period of training or losartan administration may have revealed significant correlations. Another possible limitation relates to the power of the study. Power analysis indicates that, assuming a two-tailed 5% test, 17 subjects were sufficient to detect a significant correlation of 0.65 with 80% power (20). The correlation coefficients we observed were very modest, and we feel it safe to conclude that a larger sample size would not have altered the essential lack of association between the variables compared. Finally, although L-NMMA is an inhibitor that is specific for NO production, it is possible that FMD responses are not entirely NO mediated and that we are therefore not comparing basal to stimulated NO-mediated dilation. Although several studies suggest a role for NO in the FMD response (14), a new appreciation is now beginning to emerge that more careful scrutiny of the mechanisms responsible for shear-mediated dilation in vivo is needed (29).

In summary, we have demonstrated no association between shear stress and basal NO-mediated vasodilator function, suggesting that the FMD approach may not reflect changes in basal NO function. FMD may not be an optimal surrogate for global NO dilator system function, particularly if basal NO-mediated vasodilator function is an important determinant of vascular health.

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