Evaluation of four different methods to measure endothelium-dependent vasodilation in the human peripheral circulation

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ABSTRACT

At present, several techniques exist that claim to evaluate endothelium-dependent vasodilation (EDV) in the human peripheral circulation. The present study aims to evaluate the relationships between four of these techniques. A group of 24 young, healthy subjects underwent measurements of EDV and endothelium-independent vasodilation (EIDV) in predominately resistance vessels in the forearm using the invasive forearm technique with local infusion of methacholine and sodium nitroprusside, evaluation of flow-mediated vasodilation (FMD) in the conduit brachial artery measured by ultrasound, with or without the addition of ischaemic hand exercise, and evaluation of the reduction in the relative height of the inflection point of the radial pulse wave following β2-adrenergic receptor stimulation. The reduction in the relative height of the inflection point following β2-adrenergic receptor stimulation was significantly related to both EDV and EIDV in the forearm (r = −0.41 and r = −0.42 respectively; both P < 0.05), but not to the EDV/EIDV ratio (r = −0.10). However, FMD, with or without the addition of ischaemic hand exercise, was not significantly related to the results obtained using the other two techniques (r = −0.18 to +0.13). In conclusion, the reduction in the relative height of the inflection point of the pulse wave following β2-adrenergic receptor stimulation was related to both EDV and EIDV measured by the invasive forearm technique, indicating that the pulse wave technique does not measure EDV specifically. FMD in the brachial artery, with or without ischaemic hand exercise, was not significantly related to values obtained using the other two techniques, indicating that endothelial function differs between conduit and resistance arteries, and that both of these measurements should be evaluated in future studies.

INTRODUCTION

During the last decade, much attention has been paid to the evaluation of the contribution of nitric oxide (NO) to the vasodilatory process in humans. Originally, endothelium-dependent vasodilation (EDV) was evaluated by measuring the vasomotor response to acetylcholine infused into a coronary artery during coronary angio-

Key words: blood flow, endothelium, pulse wave, ultrasound, vasodilation.

Abbreviations: EDV, endothelium-dependent vasodilation; EIDV, endothelium-independent vasodilation; EFI, endothelial function index (EDV/EIDV ratio); FBF, forearm blood flow; FMD, flow-mediated vasodilation; MCh, methacholine; l-NMMA, N-monomethyl-l-arginine; SNP, sodium nitroprusside.

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is infused into the brachial artery, and the increase in forearm blood flow (FBF) is taken as an index of EDV. Using this technique, a blunted EDV has been found in patients with coronary heart disease, hypertension, hypercholesterolaemia or diabetes [4–9]. To evaluate endothelium-independent vasodilation (EIDV), most often local infusion with sodium nitroprusside (SNP) is used. However, in contrast with most studies on the coronary circulation, the invasive forearm technique evaluates vasodilation mainly in resistance vessels.

Another method that has gained in popularity in recent years is the ultrasound-based method evaluating flow-mediated vasodilation (FMD) in the brachial artery during hyperaemia [10,11]. Like most studies in the coronary circulation, this technique evaluates vasodilation in a conduit artery. Similar to results using the invasive forearm technique, it has been shown that FMD is attenuated in patients with coronary heart disease, hypertension, hypercholesterolaemia or diabetes [12–16].

FMD declines with age [17], and low values of FMD are usually recorded in elderly subjects. Wendelhag and co-workers [18] showed recently that, by adding ischaemic hand exercise during the occlusion of the brachial artery, FMD was increased in elderly subjects. These authors suggested that adding ischaemic hand exercise would therefore be of value for quantifying endothelial dysfunction in the elderly.

It has been known for decades that nitroglycerine or other nitro-vasodilators induce a marked change in the peripheral pulse waveform, with a reduction in the characteristic ‘notch’ or point of inflection [19]. A reduction in the point of inflection could be due to a reduction in either the velocity or the amplitude of the reflected pressure wave, or both. Such an alteration would be the consequence of an increase in conduit artery compliance or of increased resistance vessel vasodilation.

More recently, it has been shown that acetylcholine lowers the height of this inflection point of the pulse wave in rabbits [20], suggesting that NO might mediate this pulse wave characteristic. In a recent human study [21] it was shown that the β2-adrenergic agonist salbutamol, known to cause vasodilation by an NO-dependent mechanism, reduced the height of the inflection point of the digital pulse wave, and that this response could be blunted by infusion of the NO blocker N-monomethyl-L-arginine (L-NMMA). Furthermore, these investigators showed that the reduction in the point of inflection of the pulse wave during inhalation of a β2-adrenergic receptor agonist was blunted in patients with Type II diabetes mellitus compared with control subjects, but the response to a nitro-vasodilator was preserved. These findings suggest that measurement of pulse wave reflection, at least in part, is NO-dependent, and might serve as an additional means to evaluate EDV.

We have found recently that EIDV evaluated by local infusion of SNP into the forearm and brachial artery vasodilation following sublingual administration of glycerol trinitrate measured by ultrasound were closely correlated [22]. However, EDV evaluated by muscarinic receptor agonist-induced vasodilation in the forearm and FMD in the brachial artery were not at all related in a subject group including patients with cardiovascular disorders [22].

The aim of the present study was to evaluate the relationships between the four different methods described above, in order to measure EDV in the peripheral circulation in a sample of young, healthy individuals.

**MATERIALS AND METHODS**

**Subjects**

The volunteer subjects consisted of 24 young, healthy students (13 men and 11 women) aged 20–25 years (mean body mass index 22.7 ± 1.8 kg/m²). None of the subjects were taking regular medication or had a history of any disease known to affect the cardiovascular system. Subjects with a history of any metabolic or other serious diseases, as well as regular smokers, were excluded from the study.

All subjects were investigated in the morning after an overnight fast. The order of the investigations was as follows: the invasive forearm technique; FMD without exercise; FMD with exercise; and assessment of the pulse wave. Rest periods of 30 min were allowed between the different measurements, a period we have found previously to be sufficient for the measured variables to return to resting values. All measurements were performed in the same (predominately the non-dominant) arm, and our pilot studies have shown that the presence of an arterial cannula does not influence the evaluation of FMD or the pulse wave.

The study was approved by the Ethics Committee of Uppsala University.

**Invasive forearm technique**

During the blood flow measurements, the subjects were supine in a quiet room maintained at a constant temperature. An arterial cannula was inserted into the brachial artery for regional infusions of methacholine (MCh) and SNP.

At least 30 min were allowed for establishing stable conditions after cannulation of the artery. Drug infusions were given over 5 min for each dose, with a 20 min washout period between the drugs. The infused dosages were 2 and 4 µg/min for MCh (evaluating EDV), and 5 and 10 µg/min for SNP (evaluating EIDV). The drugs were given in a random order at a maximal rate of 1 ml/min. Infusions were performed in one of the arms, while the contra-lateral arm served as a control.
FBF was measured in both forearms by venous occlusion plethysmography before and at the end of the different dosages of the two drugs. A mercury-in-silastic strain gauge was placed on the upper third of the forearm, which rested comfortably slightly above the level of the heart. The strain gauge was connected to a calibrated plethysmograph. Venous occlusion was achieved by a blood pressure cuff applied proximal to the elbow and inflated to 50 mmHg by a rapid cuff inflator. Evaluations of FBF were made by calculation of the mean of at least five consecutive recordings.

The reproducibility of measurements of EDV and EIDV using this technique has been evaluated previously in 10 healthy young volunteers, in whom the investigation was repeated after 2 h, and again after 3 weeks. The evaluation of both EDV and EIDV showed a variation of 5–8% in the short-term (2 h), as well as in the long-term (3 weeks), perspective [23].

We have found previously in healthy volunteers that MCh at the highest dose used in the present study induces a significant increase in forearm venous plasma nitrite and nitrate concentrations [22]. When plasma nitrite and nitrate concentrations were measured both in arterial blood and in forearm venous blood samples, the forearm release of these two breakdown products of NO was increased by more than 10-fold, indicating that the vasodilatory capacity of MCh is mediated mainly by an increase in NO production [22].

In the present study, only data obtained using the highest doses of MCh and SNP were evaluated. EDV was defined as FBF during the infusion of 4 μg/min MCh minus resting FBF divided by resting FBF. EIDV was defined as FBF during infusion of 10 μg/min SNP minus resting FBF divided by resting FBF. In addition, an endothelial function index (EFI) was calculated as the EDV/EIDV ratio. This index is supposed to reflect more specifically the contribution of the endothelium to the vasodilatory process.

**Brachial artery ultrasound technique**

Vasodilation in the brachial artery was assessed by external B-mode ultrasound imaging 2–3 cm above the elbow (Acuson instrument, equipped with a 7.0 MHz linear transducer; Acuson, Mountain View, CA, U.S.A.). Depths and gain settings were optimized to identify the lumen–vessel-wall interface, and were kept constant during each study. Images were magnified electronically with a ‘resolution box function’ in order to achieve a larger picture for measurements of the diameter. The subject rested in the supine position in a temperature-controlled room for at least 15 min before the first scan, and remained supine during the evaluation. An increase in blood flow was induced by inflation of a pneumatic cuff placed around the forearm to a pressure of 300 mmHg. The cuff was deflated 5 min later, and the artery was scanned continuously for 90 s after cuff deflation. Vessel diameter was measured before cuff inflation and at the maximal diameter, obtained 45–60 s after cuff release, in accordance with Sorensen et al. [10]. Brachial artery diameters were recorded on a super-VHS videotape during the procedures and analysed on a later occasion. The mean diameter was calculated from three to four cardiac cycles co-incident with the R-wave on the ECG. FMD was defined as the maximal brachial diameter during the hyperaemic phase following cuff release minus diameter at rest divided by diameter at rest. We also calculated the area under the curve for the change in brachial artery diameter during the hyperaemic phase, with measurements at 30, 60 and 90 s.

The coefficient of variation for repeated measurements of brachial artery diameter in our hands is 3–4% in the short term (2 h), as well as in the long term (3 weeks).

After 30 min of rest, the baseline brachial artery diameter was measured and a similar procedure was repeated, but now with the addition of ischaemic hand exercise in addition to occlusion of the forearm. After 1 min of occlusion of the forearm circulation, the subjects were instructed to pump a rubber bladder. The ischaemic hand exercise continued until ischaemic pain or exhaustion in the hand occurred (minimum 1 min). The cuff pressure was then released, and images for the measurement of the lumen diameter of the brachial artery were recorded for 3 min after cuff release [18]. Our pilot studies showed that maximal vasodilation of the brachial artery under these conditions occurs after 90 s, and measurements of brachial artery diameter at this time were therefore used when calculating FMD with addition of ischaemic hand exercise. We also calculated the area under the curve for the brachial artery diameter change during the hyperaemic phase, with measurements at 30, 60, 90, 120 and 150 s.

**Pulse wave analysis**

In the assessment of the pulse wave, a micromanometer-tipped probe (SphygmoCor; Pulse Wave Medical Ltd) was applied to the surface of the skin overlying the radial artery and the peripheral radial pulse wave was recorded continuously. For accurate recordings the micromanometer must be applied with light pressure to flatten the vessel walls, so that transmural forces within the vessel are perpendicular to the arterial surface. The mean values of at least 20 pulse waves were used for analyses. The height of the characteristic ‘notch’ or inflection point was measured in relation to the maximal systolic peak. The maximal systolic peak and the inflection point were identified by calculation of the first and second derivatives of the different parts of the pulse curve. The ratio between the height of the inflection point and the systolic height was denoted as the relative height of the inflection point; software used for this calculation was kindly supplied by the manufacturer. After a baseline recording, terbutaline was administered subcutaneously (0.25 mg,
in the upper part of the arm) and a re-evaluation of the pulse wave was performed after 15 min. We had found previously in pilot studies that the maximal reduction in the inflection point occurs after 15 min. The coefficient of variation for repeated measurements of the relative height of the inflection point is, in our hands, 8–10% both in the short term (2 h) and in the long term (3 weeks).

Statistics
Pearson’s correlation coefficient, obtained by the least-squares method, was used for the evaluation of relationships between the four different methods to measure EDV. \( P < 0.05 \) was regarded as significant.

RESULTS
Means ± S.D. of values obtained using the four different tests are given in Table 1. Correlation coefficients for the relationships between the four different tests are given in a correlation matrix in Table 2.

As can be seen in Table 2, FMD values obtained with and without ischaemic hand exercise were significantly correlated \( (r = 0.53, \ P < 0.03) \). However, results obtained using either of these methods to evaluate FMD were not significantly correlated with measurements of EDV, EIDV or EFI using the invasive technique, or with the relative height of the inflection point after \( \beta_2 \)-adrenergic receptor stimulation. Similar non-significant correlation coefficients were obtained when FMD, with or without ischaemic hand exercise, was compared with values from the invasive technique or with the relative height of the inflection point after \( \beta_2 \)-adrenergic receptor stimulation when FMD was calculated by the area under the curve method (results not shown).

On the other hand, the reduction in the relative height of the inflection point after \( \beta_2 \)-adrenergic stimulation was significantly related to both EDV and EIDV as measured by the invasive forearm technique. As can be seen in Figure 1, these relationships were very similar \( (r = -0.41, \ P < 0.05 \) for MCh-induced vasodilation; \( r = -0.42, P < 0.05 \) for SNP-induced vasodilation). Thus the relative height of the inflection point after \( \beta_2 \)-adrenergic stimulation was not related to EFI \( (r = -0.10) \) (Figure 2).

DISCUSSION
The present study shows that the reduction in the relative height of the inflection point of the pulse wave following \( \beta_2 \)-adrenergic receptor stimulation was related to both EDV and EIDV, but not to the EFI, measured by the invasive forearm technique. This indicates that the method using pulse wave analysis does not evaluate EDV.

### Table 1
Baseline values obtained using the four different methods to evaluate EDV, and the percentage changes during these methods

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline</th>
<th>Change (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial artery diameter (mm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Without hand exercise</td>
<td>3.5 ± 0.5</td>
<td>6.4 ± 4.3</td>
</tr>
<tr>
<td>With hand exercise</td>
<td>3.5 ± 0.6</td>
<td>12.6 ± 6.3</td>
</tr>
<tr>
<td>FBF (ml min(^{-1}) 100 ml(^{-1}) tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCh infusion (4 ( \mu )g/min)</td>
<td>5.1 ± 1.2</td>
<td>340 ± 144</td>
</tr>
<tr>
<td>SNP infusion (10 ( \mu )g/min)</td>
<td>5.1 ± 1.1</td>
<td>344 ± 153</td>
</tr>
<tr>
<td>Relative height of inflection point of the radial artery pulse (%)</td>
<td>45 ± 6.4</td>
<td>-37 ± 14</td>
</tr>
</tbody>
</table>

### Table 2
Pearson’s correlation coefficients for relationships between measurements obtained using the four different methods to evaluate EDV

<table>
<thead>
<tr>
<th>FMD</th>
<th>Without hand exercise</th>
<th>With hand exercise</th>
<th>EDV</th>
<th>EIDV</th>
<th>EFI</th>
<th>IP</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD (without)</td>
<td>–</td>
<td>0.53</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>FMD (with)</td>
<td>–</td>
<td>–</td>
<td>0.13</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EDV</td>
<td>–</td>
<td>–</td>
<td>0.73</td>
<td>0.15</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>EIDV</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.51</td>
<td>–</td>
</tr>
<tr>
<td>EFI</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.10</td>
</tr>
<tr>
<td>IP</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
Methods to measure endothelium-dependent vasodilation

Figure 1 Relationships between the reduction in the relative height of the inflection point at the pulse wave following β2-adrenergic stimulation and EDV ($r = 0.41$, $P < 0.05$; upper panel) and EIDV ($r = 0.42$, $P < 0.05$; lower panel) evaluated by the invasive forearm technique.

Figure 2 Lack of a significant relationship between the reduction in the relative height of the inflection point at the pulse wave following β2-adrenergic stimulation and the EFI evaluated by the invasive forearm technique ($r = 0.10$).

specifically, but rather vasodilation in general. Furthermore, FMD evaluated with or without ischaemic hand exercise was not related to the other measurements of EDV, indicating different results when endothelial function is measured after an increase in shear stress in a conduit artery and when evaluated after muscarinic receptor stimulation in resistance vessels.

The present study confirmed our previous finding that evaluation of EDV by the invasive forearm technique and of FMD measured by the brachial artery ultrasound technique were not related in a sample population that included elderly subjects with cardiovascular disorders [22]. This was a somewhat unexpected finding, as impairments in vasodilation have been found in patients with coronary heart disease, hypertension, hypercholesterolaemia and diabetes using both of these techniques [5–12,16]. On the other hand, it should be remembered that these two techniques evaluate EDV in different parts of the vasculature and following different stimuli. The invasive forearm technique evaluates EDV mainly in resistance vessels in skeletal muscle, while the brachial artery ultrasound technique measures vasodilation in a conduit artery. Furthermore, one technique uses shear stress as the stimulus for vasodilation, while the other uses a receptor-dependent mechanism. It should also be emphasized that one of the techniques measures blood flow, while the other evaluates a change in artery diameter. As these two techniques obviously evaluate different properties of the endothelium, it seems logical to include both of these established techniques in the evaluation of EDV in future studies.

The average FMD found in the present study (6.4%) was rather low compared with some studies [24,25], but similar to or even higher than those in other studies [26,27] in young healthy subjects. FMD in the present study was similar in magnitude to what we have found in other studies using a different protocol (6.6%) [22]. Thus it seems unlikely that the present protocol would have influenced the FMD results.

The addition of ischaemic hand exercise to FMD did indeed increase vasodilation in the brachial artery, but did not improve the degree of association with the other techniques. Thus this modification of the brachial ultrasound technique has to be evaluated further in order to be regarded as an alternative technique to the ‘classical’ brachial ultrasound technique.

The use of pulse wave analysis to assess the relative height of the inflection point before and after β2-adrenergic stimulation is an attractive technique, since, like the brachial ultrasound technique, it is quick to perform and does not require arterial cannulation. In previous studies [21], and our own pilot studies, it has been shown that β2-adrenergic stimulation changes the
pulse wave mainly via vasodilation of the resistance arteries, and not via an effect on conduit arteries. It was therefore not surprising that results obtained using this technique were related to those from the invasive forearm technique, using blood flow in resistance arteries as the outcome variable, but not those from the brachial ultrasound technique, which evaluates a conduit artery.

In the present study the reduction in the relative height of the inflection point after $\beta_2$-adrenergic stimulation was related to both the MCh-induced and the SNP-induced increases in blood flow in the forearm. Thus it was not related to the EFI, i.e. the ratio between the response to the muscarinic receptor agonist and the response to SNP, an index that is suggested to express the contribution of the endothelium to the vasodilatory process in the forearm. Thus from the present information it seems most likely that the reduction in the height of the inflection point following $\beta_2$-adrenergic stimulation is due to a direct effect on vascular smooth muscle tone rather than to stimulation of the endothelium. It has been shown previously that the reduction in the relative height of the inflection point following $\beta_2$-adrenergic stimulation could be blocked by co-administration of L-NMMA [21], but it seems clear that this technique needs to be investigated further before it can be used as an alternative to the other more commonly used techniques.

A limitation of the present study is that the tests cannot be performed in a random order, as the effect of subcutaneously given terbutaline is rather long lasting. Therefore, for practical reasons, it has to be administered at the end of the protocol.

In conclusion, the reduction in the relative height of the inflection point of the pulse wave following $\beta_2$-adrenergic receptor stimulation was related to both EDV and EIDV, but not to the EFI, measured by the invasive forearm technique, indicating that the method using pulse wave analysis does not evaluate EDV specifically, but rather vasodilation in general. Furthermore, FMD evaluated with or without ischaemic hand exercise was not related to the other measurements of EDV, indicating different results when endothelial function is measured after an increase in shear stress in a conduit artery and when evaluated after muscarinic receptor stimulation in resistance vessels. As these two techniques evaluate different properties of the endothelium, it seems logical to include both of them in the evaluation of EDV in future studies.

REFERENCES


Methods to measure endothelium-dependent vasodilation


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