Determinants of short-term variation in arterial flow-mediated dilatation in healthy young men

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ABSTRACT

Brachial artery FMD (flow-mediated dilatation) is widely used as a marker of systemic arterial endothelial function. FMD, however, shows considerable 25% day-to-day variation that hinders its clinical use. The reasons for this variability are poorly characterized. Therefore the present study was designed to clarify factors responsible for the hourly variation in endothelial function, including consuming a low-fat meal and circadian rhythms in endogenous hormonal levels. Brachial artery FMD, along with serum glucose, triacylglycerols (triglycerides) and levels of several hormones were measured six times per day on two separate days 1 week apart. On one day, the subjects (healthy males: n = 12, mean age, 24 years) ate a light breakfast and a standardized lunch (23.5% fat, 48.7% carbohydrate and 27.8% protein). On the other day, they had a similar breakfast after which they fasted. Postprandial FMD values (both after breakfast and after lunch) were similar to baseline FMD. FMD showed a 28% hourly variation and 27% weekly variation. Variation in plasma levels of insulin (P = 0.02) associated negatively and DHPG (3,4-dihydroxyphenylglycol) (P = 0.001), a marker of sympathetic nervous activation, associated positively with variation in FMD. The effects of DHPG and insulin on FMD were independent of changes in baseline brachial artery diameter, although DHPG was also inversely associated with baseline diameter. Eating a regular low-fat meal does not have any measurable effects on brachial artery endothelial function. These data suggest that strict requirements for fasting conditions may be unnecessary when measuring peripheral endothelial function using the ultrasound technique. Circadian variation in serum insulin and sympathetic tone are physiological determinants of endothelial function.

INTRODUCTION

Impairment of arterial endothelial function is an important early step in the atherosclerotic process [1]. Ultrasonographically measured brachial artery FMD (flow-mediated dilatation) is widely used as a marker of systemic arterial endothelial function. Previous studies have shown that the measurement of FMD is accurate and reproducible and associates closely with structural and functional coronary artery atherosclerosis [2,3].

Key words: endothelial function, flow-mediated dilatation, high-resolution ultrasound, insulin, sympathetic tone.

Abbreviations: BP, blood pressure; DHPG, 3,4-dihydroxyphenylglycol; E %, energy %; FMD, flow-mediated dilatation; GH, growth hormone; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NO, nitric oxide; SBP, systolic BP; VLDL, very-LDL.

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FMD shows, however, remarkable day-to-day variation most probably due to biological circadian rhythms and measurement error, which have hindered its clinical use in risk stratification of individual patients. The factors responsible for this substantial intra-subject variation remain to be fully characterized.

To standardize the assessment of FMD, most study groups have performed the measurements in the morning on fasting patients, which, however, limits wide-scale use of this methodology in routine clinical practice. Previous data regarding postprandial changes in endothelial function are controversial. Eating a high-fat meal has been associated with reduced endothelial function in some studies [4,5], whereas others have observed no postprandial attenuation in FMD [6]. Moreover, there are limited data available regarding the possible effects of consuming a low-fat meal on FMD [7]. This question is important, since this test is becoming more widely used and there is presently uncertainty whether study subjects could be studied in non-fasting conditions. Some previous studies have suggested that endothelial function is attenuated during the morning hours and this would contribute to the increased occurrence of acute coronary syndrome during the early morning [8]. Therefore to evaluate the effects of dietary factors, time of day and variations in serum levels of glucose, lipids, insulin, GH (growth hormone), cortisol, catecholamines and DHPG (3,4-dihydroxyphenylglycol) on endothelial function, we assessed FMD of the brachial artery at several time points before and after regular meals in healthy young men.

METHODS

We studied 12 young men aged 20–29 years, who were recruited among university students. All subjects were healthy, normotensive, normoglycaemic and lifelong non-smokers. None of the subjects was taking any medication or vitamins/antioxidant supplements. All subjects had serum cholesterol and triacylglycerol (triglyceride) levels <5 mmol/l and <2 mmol/l respectively. The study was conducted according to the Declaration of Helsinki, and the study protocol had been approved by the Joint Commission on Ethics of the Turku University and the Turku University Central Hospital. Participants gave their informed consent.

Study design

The subjects were advised to refrain from alcohol, caffeine and high-fat and high-nitrate meals and to avoid exposure to cigarette smoke for 3 days prior to and thereafter throughout the study. None of the subjects had symptoms of acute infections 1 week before or during the study. The subjects were studied on two separate days 1 week apart. The order of the two study days was assigned randomly. On one day, the subjects ate breakfast and a regular meal (23.5 % fat, 48.7 % carbohydrate and 27.8 % protein) and, on the other day, a similar breakfast after which they fasted. On both study days, FMD was measured at 08.00 hours following a 10–12-h overnight fast. A venous catheter was placed in an antecubital vein and blood samples were collected through this intravenous route, which was flushed using physiological saline following each sampling. After the baseline study, the subjects had a standardized light breakfast (two pieces of toast with cheese and a glass of water) and the ultrasound measurements were repeated at 09.00, 10.00, 11.00, 12.30 and 13.30 hours on two separate days. At each time point immediately before the ultrasound scan, BP (blood pressure) was measured and venous blood samples were drawn for glucose, triacylglycerols, insulin, cortisol, GH and catecholamine determinations. Between scans, the subjects remained seated and were allowed to take slow walks in the corridor, but otherwise exercise was forbidden. During the lunch day, a standardized low-fat meal [464 kcal (where 1 kcal = 4.184 kJ)]; 12 g of fat, 32 g of protein and 56 g of carbohydrate), consisting of 400 g of minced meat and pasta, 20 g of tomato sauce and 200 ml of water, was served at 11.30 hours. No part of the meal included preservatives. The fatty acid composition of the triacylglycerols in the meal was analysed using GLC. Of the fatty acids present, 37 % were saturated fatty acids, 58 % mono-unsaturated and approx. 5 % polyunsaturated. Of the fatty acids, 52 % was oleic acid.

Serum lipids

Serum cholesterol and triacylglycerol concentrations were determined enzymatically (Merck) using an autoanalyser (AU 510; Olympus). For the separation of the VLDL [very-LDL (low-density lipoprotein)] fraction, serum was centrifuged (18 h at 105 000 g) at a density of 1.006 g/ml. After removing VLDL, LDL was precipitated from the infranatant [HDL (high-density lipoprotein) + LDL] with dextran sulphate 500 000/MgCl₂, according to the method of Kostner [9]. Cholesterol concentrations of the fractions were assayed with an enzymatic method (Boehringer).

Plasma glucose and insulin and serum GH, cortisol and catecholamines

Plasma glucose was analysed using an enzymatic method. Insulin and GH were determined using standard immunofluorometric assays. Serum cortisol was measured using an enzyme immunoassay method. Serum noradrenaline (norepinephrine) and DHPG (3,4-dihydroxyphenylglycol) concentrations were analysed by a HPLC technique using an electrochemical detector (ESA 5100 A) and analysis kits supplied by Pharmacia LKB Biotechnology.
Determination of plasma nitrate and nitrite

NO (nitric oxide) production in plasma was monitored with the Nitric Oxide Quantitation Kit (Active Motif), according to manufacturer’s instructions. The kit is based on nitrate and nitrite determination. The absorbances were detected with a Multiscan Ascent spectrophotometer (Thermo Labsystems). The detection limit of the assay is < 1 mM.

Brachial artery physiology

All studies were performed using an Acuson Sequoia 512 mainframe (Acuson) with a 13.0 MHz linear array transducer. Brachial artery diameter was measured from B-mode ultrasound images. In all studies, scans were obtained at rest and during reactive hyperaemia. The subjects laid quietly for 10 min before every scan. The ultrasound studies were conducted in silence in a dimmed temperature-controlled clinical facility room. The left brachial artery was scanned in longitudinal section 2–15 cm above the antecubital crease. Depth and gain settings were set to optimize images of the lumen/arterial wall interface, images were magnified using a resolution box function and the operating parameters were not changed during the study. When a satisfactory transducer position was found, the position was marked on the skin and the arm remained in the same position throughout the study. A resting scan was performed and arterial flow velocity was measured using a Doppler signal. Increased flow was then induced by inflation of a pneumatic tourniquet placed around the forearm (distal to the scanned part of the artery) to a pressure of 250 mmHg for 4.5 min, and the arm remained in the same position throughout the study. When a satisfactory transducer position was found, the position was marked on the skin and the arm remained in the same position throughout the study. A resting scan was performed and arterial flow velocity was measured using a Doppler signal. Increased flow was then induced by inflation of a pneumatic tourniquet placed around the forearm (distal to the scanned part of the artery) to a pressure of 250 mmHg for 4.5 min, followed by release [10].

A second scan was taken 30–120 s after the cuff deflation. The flow velocity recording was repeated during the first 15 s after the cuff was released. All brachial ultrasound scans were recorded on super-VHS videotapes for analysis later. Vessel diameter was always measured independently by two observers who were unaware of the subject’s identity. The arterial diameter was measured at a fixed distance from an anatomic marker (e.g. a fascial plane) using ultrasonic callipers. Measurements were taken at end-diastole (incident with the R wave on a continuously recorded ECG) from the anterior to the posterior intima lumen interface (i-line). The hyperaemic diameter was measured at 45 and 60 s after cuff deflation and maximal FMD was measured at 60 s. Endothelial-independent nitrate-mediated dilatation was not assessed in the present study, because the potential accumulation of organic nitrates, due to repetitive administration, might have influenced the FMD results.

Good agreement was seen between the observers both in the measurement of the brachial artery diameter at baseline and that of the FMD percentage. The intra-class correlations were 0.99 and 0.96 respectively. The between-visits repeatability, measured as the intra-class correlation coefficient, for baseline brachial artery diameter (08.00 compared with 08.00 hours 1 week apart) was 0.90 and the CV (coefficient of variation) was 2 %, and for FMD the values were 0.57 and 24 % respectively. Brachial artery diameter was measured from the same fixed position at each time point and images of previous time points were used to ensure that the locations corresponded exactly.

Statistical methods

Results are expressed as means ± S.D. The association between FMD and biochemical or physiological variables was assessed using linear mixed modelling and repeated measures analysis of covariance. Baseline vessel diameter was included as a covariate, day (fast/lunch day) and time point were included as repeated factors, FMD as a response variable and biochemical or physiological variables as dependent variables (respectively). Univariate associations between the study variables (and also for the association between measurements at different time points) were analysed by calculating Pearson’s correlation coefficients. Multivariate analyses were done using the linear regression technique. To assess the variation in the variables between different time points with each day examined separately, linear mixed models for repeated measures were used, with FMD as a factor, time as the repeated factor and each variable (one at a time) as the dependent variable. The same model was also used when all time points were taken into account, but then the study day (fast/lunch) was also included in the model as a repeated factor. Paired Student’s t tests were used to assess the difference between the study days (first time point of both days). To assess differences between the first and second time points (baseline compared with the following time point after breakfast), the time points of the two study days were pooled and Tukey’s test was used. All statistical analysis was performed by the SAS system for Windows, version 8.2 (SAS Institute). Statistical significance was inferred at P < 0.05.

RESULTS

The baseline characteristics of the study subjects are shown in Table 1. On the fasting day, serum triacylglycerols, NO degradation products, insulin and cortisol showed a significant decreasing trend, whereas DHPG increased towards the afternoon (Table 2). Eating a light breakfast or a low-fat lunch was not associated with changes in FMD (breakfast, P = 0.27; lunch, P = 0.50; Table 3 and Figure 1). Triacylglycerols and insulin concentrations increased and GH and cortisol decreased after lunch, whereas other variables remained
unchanged (Table 2). The mean hourly variation in FMD was 28.0%, and the mean weekly variation was 27.2%. The correlation between consecutive FMD measurements with increasing time is shown in Figure 2, together with similar correlations for brachial artery baseline diameter and SBP (systolic BP) measurements for comparison. FMD measurements where more reproducible than BP measurements, but lacked reproducibility compared with brachial baseline diameter measurements (Figure 2). At baseline (38.00 hours on the fasting day), FMD was inversely associated with plasma glucose (\( r = -0.28, P = 0.02 \)), brachial artery baseline diameter (\( r = -0.25, P = 0.04 \)) and GH (\( r = -0.42, P = 0.0002 \)) and directly with DHPG (\( r = 0.34, P = 0.005 \)), but not with serum lipids (results not shown).

The variation in FMD throughout the study was inversely associated with plasma insulin (\( \beta = -0.18, P = 0.02 \)) and directly with DHPG (\( \beta = 0.95, P = 0.001 \); Figure 3) concentrations, whereas other variables had no significant associations with FMD. NO degradation products were not significantly associated with FMD (\( \beta = 0.11, P = 0.76 \)) nor was the increase in brachial artery flow during reactive hyperaemia (\( \beta = -0.33, P = 0.65 \)). DHPG was also inversely associated with baseline brachial diameter (\( \beta = -0.41, P = 0.006 \)). The associations between insulin (\( P = 0.007 \)) and DHPG (\( P = 0.039 \)) with FMD, however, persisted after controlling for baseline diameter.

**DISCUSSION**

Endothelial dysfunction is a key early event in atherosclerosis [11] that precedes and predicts the occurrence of future adverse cardiovascular events [12]. After the development of a non-invasive ultrasound method for the study of conduit artery endothelial function [13], the number of investigations in this area has increased greatly. Some authors have discussed use of the brachial artery test for endothelial function as a clinical tool for CHD (coronary heart disease) risk stratification [14]. The marked intrasubject variation in brachial FMD measurements has hindered its use in routine clinical

### Table 1 Baseline characteristics of the study subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of subjects</td>
<td>12</td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.8±3.1</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.86±0.06</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79.4±7.7</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>21.4±1.7</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.2±0.4</td>
</tr>
<tr>
<td>HDL-cholesterol (mmol/l)</td>
<td>1.4±0.3</td>
</tr>
<tr>
<td>LDL-cholesterol (mmol/l)</td>
<td>2.3±0.5</td>
</tr>
</tbody>
</table>

Values are means ± S.D. BMI, body mass index.

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The present study shows that temporal variation in arterial flow-mediated dilatation has been insufficiently characterized. The results of this study, while the factors responsible for this variability have not been identified, indicate that temporal variation in FMD is small.

Table 3: Ultrasound data on fasting and lunch days

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fasting day</th>
<th>Lunch day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>1 h</td>
</tr>
<tr>
<td>FMD (%)</td>
<td>8.4 ± 4.1</td>
<td>7.3 ± 4.3</td>
</tr>
<tr>
<td>Brachial diameter (mm)</td>
<td>3.8 ± 0.4</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>Increase in blood flow (%)</td>
<td>471 ± 228</td>
<td>588 ± 237</td>
</tr>
</tbody>
</table>

Figure 1: Brachial artery FMD on the fasting day (⃝2000) and on the lunch day (⃝2000). Values are shown as means ± S.D. Neither the time of the day nor a light lunch had any effect on endothelial function.

Figure 2: Variation in FMD, SBP and baseline brachial artery diameter with increasing time between measurement points expressed as Pearson's correlation coefficients. Variation in FMD and baseline brachial artery diameter are shown as means ± S.D. for both measurement points on the fasting day and the lunch day. The factors responsible for this variability have been insufficiently characterized. The results of this study, while the factors responsible for this variability have been insufficiently characterized, indicate that temporal variation in FMD is small.

Figure 3: Association between FMD and DHPG including all time points during the study.
plasma insulin concentration and sympathetic nervous activation, assessed as serum DHPG, are physiological regulators of FMD, whereas time of day and ingestion of light low-fat meals have negligible effects on test results.

Previous observations regarding postprandial changes in endothelial function are controversial. Some investigators have associated ingestion of a high-fat meal with an acute impairment of the FMD response [4,5], but this attenuation in FMD after a fatty meal may result from postprandial arterial dilatation and may not reflect disturbances in the NO pathway [15]. A previous study [16] has shown impaired endothelial function after a meal rich in cooking fat, but not after a meal without it, despite similar increases in postprandial triacylglycerol levels, suggesting that oxidatively modified fats may deteriorate endothelial function. In the study by Steer et al. [7], consumption of a high-fat meal [34 E % (energy %)] acutely attenuated, a low-fat meal (20 E %) had no effects and a minimal-fat meal (3 E %) ameliorated endothelial function. In the present study, we examined the effects of a regular light breakfast and a low-fat lunch on brachial FMD. The light lunch did not induce significant changes in arterial basal tone or FMD, despite small but statistically significant contemporaneous increases in triacylglycerols and insulin concentrations. These results show that consumption of a low-fat meal has no significant effects on arterial endothelial function. This finding has significant implications in regard of the clinical use of FMD, as most groups have performed the studies only in fasting patients and limited data are available on the effects of eating on FMD. The results reported in the present study suggest that strict requirements of fasting during the FMD assessment are not imperative.

In the present study, FMD had a mean hour-to-hour variation of 28 % and a mean weekly variation of 27 %, which are in line with previous estimates [17–19]. Circadian rhythms in levels of endogenous hormones and metabolites, as well as in sympathetic tone, have been implicated as being responsible for diurnal variation in FMD, although data on these factors are scarce. The novel finding of the present study is that plasma insulin concentrations and alterations in sympathetic nervous activity measured as serum DHPG, an intraneuronal metabolite of noradrenaline and the substance most excessively released to the circulation from sympathetic nerve endings during activation, are significant determinants of FMD variation in healthy young men during physiological conditions. Previous studies have shown that hyperinsulinaemia induces endothelial dysfunction, although, when measured as FMD, the attenuation can be partly explained by insulin-mediated increases in baseline arterial diameter [6]. The association between DHPG and FMD is interesting. Endothelial production and release of NO are associated with sympathetic vasoconstrictor activity via a feedback loop. The endothelium has been reported to inhibit the release of noradrenaline from sympathetic nerve terminals and to ameliorate noradrenaline metabolism in the rabbit carotid artery [20], whereas NO release counteracts adrenergic vasoconstriction [21,22]. An increase in sympathetic tone may enhance vascular NO release by increasing arterial shear stress and also through direct agonistic stimulation [23,24]. It may be assumed that the variability in FMD in disease states, such as diabetes or heart failure, exceeds the variation observed in healthy individuals. Our results offer no data on the variability in the above-mentioned disease states; however, it is presumed that, as consuming a meal may lead to more marked increases in glucose and NEFA (non-esterified fatty acid) levels in Type II diabetics, these factors might influence FMD more than in healthy individuals with normal glucose tolerance. Moreover, in individuals with heart failure, the presence of increased sympathetic tone may be thought to influence FMD. In a previous study by West and coworkers [19], the day-to-day variation in FMD in Type II diabetics was, however, reported to be comparable with healthy individuals, although individuals with greater variations in glucose and insulin levels also had greater FMD variation.

The plasma levels of NO degradation products (nitrite and nitrate) showed significant hourly variation with highest levels in the morning and lowest in the afternoon, but were not significantly associated with FMD. In a previous study in healthy pre-menopausal women [25], the urinary excretion or plasma levels of nitrate did not display diurnal variation or associate with FMD. NO degradation products have been shown to decrease in the postprandial state [26]. The lack of an association between FMD and NO degradation products, understandable as the levels of nitrate and nitrite, are closely dependent on their dietary intake and are not specific markers of NO degradation. Serum noradrenaline was not associated with arterial diameter or FMD in the present study. Only small amounts of noradrenaline are released to the circulation during sympathetic activation compared with DHPG, which therefore serves as a more sensitive marker of sympathetic tone than noradrenaline [27]. In a previous study by Hijmering et al. [28], increased sympathetic stimulation, induced by baroreceptor unloading, using a lower-body-negative-pressure box was associated with attenuation of endothelial function. Our present study partly contradicts these results by showing that moderate sympathetic tone in healthy young men in the resting state may be associated with increased endothelium-dependent arterial dilatation. Using a lower-body-negative-pressure box may be expected to produce a marked increase in sympathetic stimulation compared with the normal resting state variation in healthy individuals. Although DHPG associated with FMD, it is apparent that in clinical practice the measurement of DHPG levels does not help in interpreting of FMD results nor can the
FMD results be corrected using DHPG. However, the clinical implication of this observation is that necessary measurements should be undertaken to control sympathetic nervous activity during FMD testing, e.g. by using standardized study conditions, such as quiet and dimmed laboratory facilities. The moderate temporal variations in levels of triacylglycerols, glucose, cortisol, GH or noradrenaline were not associated with variation in FMD.

In the present study, no significant hourly variation was observed in FMD. Some previous studies have demonstrated lower FMD values in the morning compared with the afternoon and evening and have suggested that the increased occurrence of acute cardiovascular events might be a result of a physiological attenuation in endothelial function during early morning hours [8,29]. Our present results, however, demonstrate that, at least in healthy subjects studied in a controlled environment and during physiological conditions, no morning attenuation in FMD was observed compared with afternoon FMD values. In the study by Etsuda and co-workers [8], FMD was higher at 17.00 hours compared with values observed before noon. If there is significant diurnal variation in FMD, it does not seem to be apparent during the morning and early afternoon.

The reactive hyperaemia response (flow increase after cuff release) seemed to increase towards the afternoon compared with the morning values. The reason for this increase remains unclear, although the finding was consistently observed on both study days. However, similar to that which we have shown previously [30], this increase in reactive hyperaemia was not associated with increases in the FMD response.

Our present study does have some limitations. The study included a relatively small number of subjects and only males. Therefore it is not clear how directly the present results can be generalized to the assessment of FMD in women, in whom the menstrual cycle has been shown to be an important determinant of FMD [31]. However, the study included healthy young men within a narrow age range, the study of which may provide unique data on the physiological determinants of FMD variation that is unobscured by chronic diseases and lifestyle influences. We only studied the subjects between 08.00 and 14.00 hours, which represent the potential schedule for the FMD test if it was to be used for risk stratification purposes in routine clinical practice. Furthermore, the subjects were studied on two separate days with 1 week between the studies.

In summary, temporal variation in FMD during the daytime hours is partly explained by alterations in plasma insulin concentrations and sympathetic tone, whereas ingestion of light low-fat meals or time of measurement between 08.00 and 14.00 hours does not significantly affect the test results.

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