Diurnal variation of flow-mediated vasodilation in healthy premenopausal women

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Diurnal variation of flow-mediated vasodilation in healthy premenopausal women. Am J Physiol Heart Circ Physiol 279: H2720–H2725, 2000.—The present study was designed to test the hypothesis of a diurnal variation in endothelial function. Sixteen healthy, nonsmoking women were studied, each on four occasions during one 24-h period (2:00 PM, 8:00 PM, 2:00 AM, and 8:00 AM). Endothelial function was assessed by ultrasound determinations of flow-mediated vasodilation (FMD%) in the brachial artery. FMD% was contrasted with endothelium-independent vasodilation, i.e., nitroglycerine-induced vasodilation (NTG%). Additionally, plasma concentrations and urinary excretion of nitrate and cGMP were analyzed. FMD% and NTG% displayed diurnal, albeit not parallel, patterns of variation. Whereas FMD% gradually increased from 2:00 PM and peaked at 2:00 AM (means ± SE: 3.1 ± 0.4, 4.4 ± 0.4, 5.1 ± 0.9, and 3.9 ± 0.8%), the NTG% demonstrated a nadir at 2:00 AM. Plasma levels and urinary excretion of nitrate and cGMP did not display diurnal variation and no clear association with the variations seen in FMD% and NTG%. This study demonstrates a diurnal variation in both endothelium-dependent and -independent vasodilation in the brachial artery of healthy women. The background and possible implication of such a variation require further studies.

Keywords: nitric oxide; nitrate; guanosine 3′,5′-cyclic monophosphate; endothelium; catecholamines

THE INCIDENCE OF UNSTABLE ANGINA, myocardial infarction, stroke, sudden cardiac death, and pulmonary thromboembolism all display diurnal variation with an accumulation in the morning (20, 22). Proposed as underlying pathophysiological triggers of the increased cardiovascular vulnerability at this time of day have been morning hypercoagulability, hypofibrinolysis, and increased vascular tone (1, 3, 9, 23, 29). The diatomic molecule nitric oxide (NO), produced by endothelial cells, has been shown to induce relaxation of smooth muscle cells (21) and to inhibit platelet adhesiveness and aggregability (10, 24, 25). NO thus affects both vascular tone and platelet activity. Indeed, coronary segments with dysfunctional endothelium have been reported to display morning exaggeration in basal tone and in their constrictor response to acetylcholine, whereas segments with normally functioning endothelium showed no such variation (6). Hypothetically, this could imply that a healthy endothelium is involved in countering diurnal variations in vasoconstricting forces by altering its production of NO by time of day. The aim of this study was, consequently, to evaluate endothelial-derived NO production at different points of time during a 24-h period.

Direct measurements of NO production in vivo are complicated by the free radical properties and, consequently, rapid inactivation of the molecule. Therefore, indirect measurements of NO formation or activity have to be used. In this study, flow-mediated vasodilation (FMD%) and nitroglycerine-induced vasodilation (NTG%) of the brachial artery were determined by ultrasound. The assessment of FMD% was previously reported to adequately reflect NO-mediated vasodilation (13, 17). Additionally, analyses in plasma and urine of nitrate, the major metabolite of NO (34), and cGMP, the second messenger to NO, were performed. Sixteen healthy women were examined with these techniques, each on four occasions during a 24-h period (2:00 PM, 8:00 PM, 2:00 AM, and 8:00 AM), to test the hypothesis of a possible diurnal variation in endothelial function.

SUBJECTS AND METHODS

Study Population

Sixteen healthy nonsmoking women aged 19–31 yr were studied. None of the participants were using any medication, including oral contraceptives. One study subject was excluded from statistical analyses of plasma and urinary excretion of nitrate. Her plasma level of nitrate was increased twofold on the second blood sampling occasion at 7:45 PM compared with the first occasion at 1:45 PM, followed by a gradual fall in concentrations on the third and fourth sampling occasions. From our experience (15), this indicates intake of nitrate-rich food, which was against the given instructions. Another study participant was excluded from statistical analyses of plasma norepinephrine due to hemoconcentration of one blood sample.

The participants were included in the study after verbal and written informed consent had been given. The study was approved by the Göteborg University Ethics Committee for Clinical Research.

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Study Design

All participants were instructed to avoid strenuous exercise and to keep a nitrate/nitrite-poorn diet during the 48 h preceding the study period and 24 h of each study occasion (14, 15). In addition, the subjects had to refrain from intake of caffeine-containing beverages during the study period.

The procedures took place in March 1997 in a temperature-controlled room (22–24°C). During four 24-h periods, the 16 study subjects were examined in groups of four. Because there were two investigators, the subjects were paired and examined by the same investigator throughout the 24-h period. The times of 2:00 PM, 8:00 PM, 2:00 AM, and 8:00 AM were selected as study points for the diurnal evaluation (a more detailed time schedule is given below). The study subjects could leave the hospital between the first and second examination but were otherwise inside the hospital. To not disturb the normal sleeping pattern, measures were taken to ensure a relaxed atmosphere. The study subjects slept for ~8 h, from 11:00–11:30 PM to 7:30 AM.

An intravenous cannula was inserted into an antecubital vein of the subject's left arm on arrival at the laboratory at 1:15 PM, i.e., 30 min before the first blood sample was collected. Blood was drawn from the intravenous cannula after at least 10 min of supine rest with 6-h intervals starting at 1:45 PM and ending at 7:45 AM. The first 5 ml of blood was discarded. After each blood sampling, the cannula was flushed with 10 ml of a solution of 0.3 ml 5,000 IU heparin/100 ml saline. Urine was collected in four portions; the first portion was collected between 7:30 AM and 1:30 PM, the second between 1:30 PM and 7:30 PM, the third between 7:30 AM and ~2:45 AM, i.e., after the third ultrasound examination was finished (in order not to alert the study subjects before the examination), and the fourth portion between ~2:45 AM and 7:30 AM. Ultrasound examinations were performed to determine FMD% and NTG%. These examinations were started at 2:00 PM, 8:00 PM, 2:00 AM, and 8:00 AM. The examinations of FMD% and NTG% lasted for ~40 min per study subject. The ultrasound examinations went on for 1 h 20 min after the points of time 2:00 PM, 8:00 PM, 2:00 AM, and 8:00 AM. One of the two study subjects (per investigator) was examined first on every one of the four study points. Blood pressure was controlled before the ultrasound examinations.

For clarity reasons, the points of time for biochemical and ultrasound analyses are presented as 2:00 PM, 8:00 PM, 2:00 AM, and 8:00 AM.

Flow-mediated vasodilation. B mode ultrasound images of the right brachial artery were obtained in longitudinal sections by use of identical 10.0-MHz linear phased array transducers and two Acuson 128 XP/10. After finding a satisfactory transducer position 6–10 cm proximal to the antecubital fossa, the transducer was fixed using a robot arm (Kanetec MB-FX) and kept in the same position throughout the examination. The skin was marked to ensure the same position on all four examinations. After a baseline scan had been recorded, arterial occlusion was elicited by a blood pressure cuff applied distally to the antecubital fossa and inflated to 250 mmHg for 4.5 min causing reactive hyperemia on its release (16). A second scan was recorded continuously from 30 s before to 90 s after cuff deflation. Doppler-derived blood flow measurements were recorded using a pulsed Doppler signal with angle correction in the center of the artery. Recordings of flow were performed at baseline and again during the first 15 s after cuff deflation.

Data evaluation. Brachial artery diameters were measured off-line by two observers from frozen images recorded on super-VHS videotape. Each investigator used three videotapes that were exchanged after each examination. The purpose was to scatter the investigations on the tapes to minimize the risk of bias on analysis. The computerized analyzing system and techniques of measurements have been described previously (33). Briefly, the system consists of a video tape player connected to a personal computer with an attached frame grabber and digitizer. Measurements were performed on four frozen images incident with the R wave of consecutive cardiac cycles on the electrocardiogram. The four measurements were later averaged. With the aid of the digitizer, a cursor was manually traced along 10 mm of the trailing edge of the near wall interface to the leading edge of the far wall interface. To assess flow-mediated diameter changes, measurements were made at baseline and 60 s after cuff deflation. Flow-mediated diameter (D) increase (FMD%) was defined as \( \Delta D/D_{\text{baseline}} \times 100 \). The results of the two observers were averaged. Blood flow was calculated from the velocity time integral of the Doppler flow velocity and the calculated transverse vessel area and heart rate. Maximum blood flow was measured 10 s after the release of the blood pressure cuff.

Reproducibility. Reproducibility and variability of brachial artery measurements were calculated for the two observers and were as follows: baseline diameter: reproducibility, 3.43 ± 0.09 versus 3.40 ± 0.08 mm \((r=0.96; P < 0.001)\); coefficient of variation (CV%) 2.91; maximal diameter during flow-dependent vasodilation: reproducibility, 3.57 ± 0.09 versus 3.56 ± 0.08 mm \((r=0.96; P < 0.001)\); CV% 2.86; percent change of brachial artery diameter during FMD%: reproducibility, 4.02 ± 0.64 versus 4.76 ± 0.74% \((r=0.84; P < 0.001)\); CV% 27.8.

NTG-induced vasodilation. Examination of NTG% was commenced 13 min after the release of the blood pressure cuff by recording a new baseline scan. Subsequently a 0.5-mg tablet of nitroglycerin was administered sublingually to the study subject. A new scan was recorded 3–4 min after nitroglycerin administration. The same analyzing procedure was made as described above.

Analysis of nitrate in blood and urine. Venous blood (5 ml) was drawn into heparin tubes. Plasma was separated by centrifugation at 3,000 rpm for 10 min and stored at −20°C until analysis. On analysis, a known volume of plasma/urine was added with a known amount of K\(^{15}\)NO\(_3\) (Sigma, St. Louis, MO) as an internal standard. Subsequently, 50 \(\mu\)l of the plasma sample were added to an Eppendorf tube that had been kept at −80°C for 20 min containing a mixture of 750 \(\mu\)l of benzene and 120 \(\mu\)l of trifluoro-methane-sulphonic acid (TFMSA) (Sigma). Upon urine analyses, the proportions were 20 \(\mu\)l of urine sample, 1,000 \(\mu\)l benzene, and 50 \(\mu\)l TFMSA.) The tube was shaken for 30 min, allowing endogenous and added nitrate to be converted to nitrobenzene. After centrifugation, 500–600 \(\mu\)l of the organic phase were separated and briefly washed with 150 \(\mu\)l of 0.5 M Na\(_2\)CO\(_3\). Subsequently, 1 \(\mu\)l of the organic phase was injected into a temperature program (60–120°C)-controlled Varian 3400 gas chromatograph equipped with a 30-m XTI-5 capillary column. The gas chromatograph was connected to a Varian Saturn mass spectrometer operated in the positive ion/chemical ionization mode, selectively monitoring mass equivalent 124 for endogenous nitrate and mass equivalent 125 for the \(^{15}\)N-labeled internal standard. Methane was used as reagent gas. The detection limit for endogenous nitrate was 0.1 \(\mu\)M.
Analysis of cGMP in blood and urine. cGMP levels in plasma and urine were determined using a commercial 125I-labeled radioimmunoassay kit (RPA 525, Amersham International). Blood was collected in precooled EDTA tubes (7 ml) and kept on crushed ice. The tubes were immediately centrifuged for 10 min at 1,500 g (4°C), and plasma was removed. Plasma was diluted, and plasma proteins were precipitated by adding 1 ml of distilled water and 2 ml of triethylamine to 1 ml of plasma. After 20 s of shaking, the diluted plasma was centrifuged for 10 min at 1,500 g (4°C), and the supernatant was removed and stored at −70°C. Before analyses, the protein-depleted plasma was concentrated using SAX (Strong Anion Exchanger) columns (RPA 1918, Amersham International). The eluate was dried in a water bath (37°C) under a stream of nitrogen and afterward diluted with 1 ml of sodium azide buffer (assay buffer). On analysis, the solution was diluted 1:5 in assay buffer. cGMP in plasma was then determined according to the acetylation protocol.

Urine was diluted 1:40 in assay buffer and analyzed according to the nonacetylation protocol.

Analysis of epinephrine and norepinephrine in plasma. One to two milliliters of plasma was used for analysis. Catecholamines and other cis-diol metabolites were selectively isolated from plasma at pH 8.6 with the aid of acid-washed aluminium oxide. Thereafter, the amines were eluted from the aluminium oxide using a mixture of acetic acid and phosphoric acid. The sample was then analyzed with HPLC on a reverse-phase column. The catecholamines were selectively detected on an electrochemical detector within a cell with two porous graphite electrodes connected in series. The method is quantitative and is linear in the interval of 0.1–400 pmol (7).

Analysis of lipids and lipoproteins in serum. Cholesterol and triglyceride levels were determined by fully enzymatic techniques by using a Gilford System 3500 Autoanalyser (Gilford Instruments, Oberlin, OH). High-density lipoprotein cholesterol (HDL-C) was determined after precipitation of apolipoprotein B-containing lipoproteins with manganese chloride and heparin. Low-density lipoprotein cholesterol (LDL-C) was calculated according to Friedewald et al. (8).

Statistical Methods

One-way ANOVA was used to determine the effect of time of day. When an overall effect of time was found, subsequent contrast analysis was performed. Linear regression analysis (Pearson) was performed to evaluate reproducibility. When CV% for FMD% was calculated, SD was determined using the formula $SD = \sqrt{\frac{\sum(x_1 - x_2)^2}{2N}}$ (2). $P < 0.05$ was regarded as statistically significant. Background tabulated data are presented as means ± SD to illustrate the variability in the sample, whereas main study variables in the figures are presented as means ± SE to quantify the uncertainty in the estimate of the true mean.

RESULTS

The procedures were well tolerated by the participants. At 2:00 AM, the majority of the study subjects were asleep or in a slumber during the blood sample collection and ultrasound examinations.

Systolic blood pressure varied significantly during the 24 h ($P < 0.01$), displaying a marked nadir at 2:00 AM ($P < 0.001$) and subsequent increase in the morning ($P < 0.01$). Diastolic blood pressure and heart rate did not show any diurnal variation (Table 1). Serum cortisol concentrations varied significantly with the characteristic morning increase ($P < 0.001$) and displayed a significant difference ($P < 0.001$) between each time point except between 8:00 PM and 2:00 AM (Table 1). A diurnal pattern was detected in plasma norepinephrine ($P < 0.001$), with higher concentrations at 2:00 PM and 8:00 PM and lower concentrations at 2:00 AM and 8:00 AM ($P < 0.001$). Plasma epinephrine did not display any variation over the 24 h (Table 1). Diurnal variations were noted in the levels of serum lipids and lipoproteins. Thus total cholesterol, LDL-C, and HDL-C concentrations were significantly lower at 2:00 AM compared with daytime values ($P < 0.05$ to $P < 0.001$), with the exception that total cholesterol did not differ between 2:00 AM and 8:00 AM. An opposite pattern was noted for serum triglycerides, the concentrations of which were higher ($P < 0.01$) at 2:00 AM (as well as at 8:00 PM) compared with 2:00 PM and 8:00 AM. Although mean values for FMD% varied in the same direction as serum triglyceride levels and opposite the HDL-C levels, there was no significant correlation between change in FMD% and change in serum lipid or lipoprotein levels.

FMD% displayed a diurnal variation (means ± SE: $3.1 ± 0.4, 4.4 ± 0.4, 5.1 ± 0.9, 3.9 ± 0.8; P < 0.05$) and increased gradually from 2:00 PM to 2:00 AM ($P < 0.01$). NTG% also demonstrated diurnal variation ($P < 0.05$) with a biphasic pattern, i.e., a weak ten-

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<tr>
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<td>S-cortisol, µM</td>
<td>255 ± 88</td>
<td>134 ± 67</td>
<td>103 ± 74</td>
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<td>P-NE, nM (n = 15)</td>
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<td>1.68 ± 1.02</td>
<td>0.98 ± 0.52</td>
<td>0.98 ± 0.37‡</td>
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<td>P-E, nM</td>
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<td>S-cholesterol, µM (n = 12)</td>
<td>4.58 ± 1.24</td>
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<td>4.35 ± 1.22</td>
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<td>S-triglycerides, µM (n = 12)</td>
<td>0.81 ± 0.35</td>
<td>1.13 ± 0.57</td>
<td>1.04 ± 0.65</td>
<td>0.82 ± 0.41†</td>
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<td>HDL-C, µM (n = 12)</td>
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<td>1.26 ± 0.17</td>
<td>1.34 ± 0.12†</td>
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<td>LDL-C, µM (n = 12)</td>
<td>2.83 ± 1.09</td>
<td>2.86 ± 0.97</td>
<td>2.63 ± 0.99</td>
<td>2.80 ± 1.09*</td>
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Values are means ± SD. SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; HR, heart rate; LDL-C, low-density lipoprotein cholesterol; P-NE, plasma norepinephrine; P-E, plasma epinephrine; S-, serum. Overall significance of diurnal variation: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. https://ajpheart.physiology.org/ DOI: 10.1152/ajpheart.00233.2017
enzy to increase 2:00 PM–8:00 PM ($P = 0.09$), a tendency to decrease 8:00 PM–2:00 AM ($P = 0.07$), and a significant increase 2:00 AM–8:00 AM ($P < 0.05$). At 2:00 PM, NTG% was significantly lower than at 8:00 AM ($P < 0.05$) (Fig. 1A). A significant ($P < 0.001$) variation was detected in basal vessel diameter with a larger diameter at 2:00 AM compared with 8:00 PM ($P < 0.01$) and 8:00 AM ($P < 0.001$) (Fig. 1B). Blood flow increase in response to hyperemia did not display any diurnal variation (mean $516 \pm 35$ ml/min), whereas shear rate increase (shear rate was calculated using the basal diameter and volume flow) did ($P < 0.01$). The diurnal patterns of shear rate increase and FMD% were, however, not parallel (Fig. 1B).

**DISCUSSION**

The major new finding in the present study was the diurnal variation of FMD%. FMD% was previously...
shown to adequately reflect release of endothelial-derived NO in response to flow (13, 18). This would seemingly imply a diurnal variation in NO production in response to flow, with its greatest capacity at night. However, the analyses of NTG%, nitrate, and cGMP did not support such a concept.

It was considered important for the outcome of the study that the subjects did display a normal diurnal rhythm. Serum levels of cortisol, displaying a marked elevation in the morning, have a diurnal variation that is well established (32). Serum cortisol was, therefore, measured and was found to display a distinct diurnal variation in all the study subjects. Because the study subjects also reported to have slept well during the night of the study, we assume that the study conditions did not substantially disturb the normal diurnal rhythm.

FMD% has been shown to be related to HDL cholesterol (26, 30), but we did not find any significant relation between change in FMD% and diurnal change in lipids or lipoproteins. Reproducibility for brachial artery inner diameter measurements is excellent, with a CV% around 3% in our study and in that by Hata et al. (11). Previous methodological studies indicated the possibility to detect within-subject changes in FMD% in the order of 2% provided a sample size similar to the current investigation (27). Not only endothelium-dependent (i.e., FMD%), but also endothelium-independent, i.e., NTG-induced, vasodilation (NTG%) were found to display diurnal variation. However, the pattern of NTG% differed from the diurnal pattern of FMD%; instead of displaying a maximum between 2:00 and 3:00 AM, NTG% was low at this point of time. This indicates that smooth muscle diurnal variation in sensitivity to NTG, and thus to NO, is not sufficient to explain the diurnal variation in FMD%.

NO production has several links to sympathetic vasoconstrictor activity. Thus the endothelium has been reported to inhibit the release of norepinephrine from sympathetic nerve terminals and also to increase norepinephrine metabolism in the rabbit carotid artery (5). Furthermore, the molecule is considered to counteract adrenergic vasoconstriction (4, 28). It has even been proposed that an important mechanism of vasodilation by NO is inhibition of peripheral sympathetic vasoconstriction (35). An increase in sympathetic tone has also been proposed to enhance vascular NO release by inducing increased shear stress and by direct agonistic stimulation (12, 31). These data suggest that NO, in several respects, acts as a feedback inhibitor of sympathetic vasoconstrictor activity.

Diurnal variations of vascular tone, α-sympathetic vasoconstrictor activity, and catecholamines have previously been reported, with increases of all these variables in the morning (19, 23). In this study, systolic blood pressure and basal vessel diameter displayed a diurnal variation, with lower systolic blood pressure and a larger vessel diameter at night. At the same time, plasma norepinephrine concentrations fell markedly. Taken together, this confirms that the sympathetic activity of the study subjects was lower at night (2:00–3:00 AM). Hence, one or several of the above-mentioned mechanisms implicating a NO-induced feedback inhibition of sympathetic vasoconstriction was probably not responsible for the increased FMD% at 2:00–3:00 AM presently observed. Rather, one may speculate that FMD% might have increased at night as a result of the concurrent attenuated sympathetic activity.

It is possible that the increased formation of endothelial NO in response to flow might cause greater relaxation of the smooth muscle cells when less counteracted by perivascular sympathetic nerves. However, the fact that the brachial artery responded less to nitroglycerin at 2:00–3:00 AM speaks against an increased responsiveness of the smooth muscle cells to NO at night. Thus it is less likely that the increased FMD% at night (2:00–3:00 AM) was due only to decreased sympathetic activity.

One alternative explanation for the diurnal pattern of FMD% might have been a difference in shear forces acting on the vessel wall at different times of day with elevated shear stress increases when FMD% was higher and vice versa. However, neither blood flow increases nor shear rate increases in response to hyperemia displayed a pattern that could explain the diurnal rhythm of FMD%.

Biochemical assessment of NO production at the four points of time was reflected by determinations of nitrate and cGMP in plasma and urine. A diurnal variation could not be detected in these variables. Thus the concept that the observed diurnal pattern of FMD% reflected a diurnal variation in NO production was not supported despite the fact that we have found a low variability of nitrate levels in subjects on a nitrate/nitrite-poor diet (15).

Our study gives no clear-cut evidence for a variation of NO production by time of day. Yet, the different rhythms of FMD% and NTG% over the 24 h might imply such a variation. The question whether NO-production displays diurnal variation can only be answered by further studies, possibly by administration of NO synthase blockers to investigate whether this abolishes the diurnal variation of FMD%.

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