Diurnal Variation in Endothelium-Dependent Vasodilatation Is Not Apparent in Coronary Artery Disease

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Background—Acute coronary syndromes present with an increased incidence from 6:00 AM to 12:00 noon. Whether endothelial function follows a diurnal rhythm and whether this rhythm is impaired in coronary artery disease (CAD) has not previously been studied.

Methods and Results—Diurnal variation in endothelium-dependent vasodilatation was examined in 10 CAD patients and 10 control subjects. Forearm blood flow responses to acetylcholine, sodium nitroprusside, and N\textsuperscript{G}-monomethyl-L-arginine were determined by plethysmography at 8:00 AM, 2:00 PM, and 8:00 PM. Heart rate, blood pressure, plasma cortisol, and inflammatory markers were also determined. Heart rate and the low-frequency component of heart rate variability were greatest in the morning in control subjects, suggesting a diurnal variation in sympathetic activity. Basal forearm blood flows were significantly reduced in control subjects at 8:00 PM compared with 8:00 AM and 2:00 PM (1.2±0.2 versus 2.1±0.2 [8:00 AM] and 2.1±0.3 [2:00 PM] mL · 100 mL\textsuperscript{-1} · min\textsuperscript{-1}; \(P<0.05\)) but unchanged in the CAD group. Acetylcholine (37 μg/min) responses were greater at 8:00 AM than at 8:00 PM in control subjects (12.5±3.7 versus 19.6±2.9 mL · 100 mL\textsuperscript{-1} · min\textsuperscript{-1}, respectively; \(P<0.05\)), but these responses were not time dependent in the CAD group. Responses to sodium nitroprusside were similar at all time points and between those with and without CAD.

Conclusions—Thus, normal volunteers have a diurnal variation in their endothelium-dependent vasodilatation that may counteract other, potentially adverse, diurnal variations in hemodynamic and other parameters. In contrast, CAD patients who had presented with acute coronary syndromes showed a loss of this protective mechanism. (Circulation. 2001;103: 806-812.)

Key Words: endothelium  ■  vasodilation  ■  circadian rhythm  ■  coronary disease

There are numerous studies demonstrating altered endothelium function in patients with coronary artery disease (CAD)\textsuperscript{1-4} and also in those at increased risk of CAD.\textsuperscript{5} The majority of these studies have evaluated vasodilator responses to pharmacological agonists or hemodynamic changes (such as shear stress) that elicit vasodilatation through endothelium-derived processes. The links between endothelial dysfunction, atherosclerosis, and clinical presentation could result from a number of the consequences of disordered endothelium function. Thus, in addition to shifting the balance between vasoconstriction and vasodilatation, there may be important effects on cell adhesion, thrombosis, and fibrinolysis.\textsuperscript{6,7}

A number of cardiovascular diseases show a circadian variation in their times of presentation. Thus, the onset of myocardial infarction is \(\approx1.3\) times greater in the period from 6:00 AM to 12:00 noon than the average occurrence during the remainder of the day.\textsuperscript{8} A similar diurnal variation has been found for the presentation of cerebrovascular events,\textsuperscript{9} sudden cardiac death,\textsuperscript{10} and pulmonary emboli.\textsuperscript{11} An increased incidence of cardiovascular events during the morning could be due to an increase in pro-occlusive factors during this time or a reduction in protective mechanisms. Hemodynamic changes may increase the chance of plaque rupture in coronary arteries harboring atheromatous plaques. Increases in coagulation and decreases in fibrinolysis may also contribute to the circadian pattern, and indeed 1 study shows blood to be hyperthrombotic and hypofibrinolytic at 8:00 AM versus 8:00 PM.\textsuperscript{12} Platelets have also been shown to be hyperaggregable in the early morning.\textsuperscript{13} As discussed, there is evidence to implicate endothelial dysfunction in the development and presentation of CAD. However, information on diurnal variation in endothelial function is not available. Alteration in endothelium function during the day may contribute to the overall risk of CAD as well as to its time of presentation.

In the present study, we report on diurnal variation in endothelium-dependent vasodilator function in normal subjects and in patients with CAD. In addition, we have documented a number of other parameters previously shown to exhibit diurnal variation, including an assessment of sympathetic activity, as well as inflammatory markers recently shown to relate to endothelial function.\textsuperscript{14}
Methods

Subjects
Ten male patients with angiographically proven CAD (aged 51.0 ± 3.4 years) and a control group (n = 10, all males, aged 56.0 ± 2.1 years) were recruited for the study. All CAD patients were studied at least 3 months after an admission to the hospital with either an acute myocardial infarction or unstable angina. Five patients had normal left ventricular function; the remainder had, at most, moderate reduction in function. Six of the patients had predominantly single-vessel disease treated with angioplasty/stent; 1 patient had severe triple-vessel disease and underwent CABG. The remaining 3 patients had at least a 50% stenosis in the culprit vessel and were treated medically. The healthy volunteers had no history of cardiovascular disease.

The present study was approved by the Alfred Group of Hospitals Ethics Committee, and written informed consent was obtained from all participants.

Experimental Protocol
Subjects were studied at 8:00 AM and 2:00 PM and at 2:00 PM and 8:00 PM on 2 occasions separated by at least 2 weeks. Subjects were randomly allotted to undergo their first study beginning at either 8:00 AM or 2:00 PM. Vascular reactivity, spectral analysis, biochemical parameters, and inflammatory markers were monitored at each time point. Lipid-lowering, antihypertensive, and long-acting antianginal medication was stopped 2 weeks and aspirin was stopped 5 days before each study day.

On arrival, the brachial artery in the nondominant arm was cannulated (3F, 5-cm catheter, Cook) under aseptic conditions, and local anesthesia (1% lidocaine) was administered for intra-arterial pressure recording and drug infusion. Arterial blood pressure was recorded with an AE 840 physiological pressure transducer throughout the course of each experiment. Lead II of the ECG was monitored throughout. After arterial cannulation, a 10-minute rest period was followed by 20 minutes of continuous pulse and blood pressure recordings for spectral analysis of heart rate variability (see below). Arterial blood was then withdrawn for cholesterol, triglyceride, glucose, cortisol, C-reactive protein (CRP), and interleukin (IL)-6 assessment.

Forearm Venous Occlusion Plethysmography
Forearm blood flow was measured by use of venous occlusion plethysmography with a double-strand alloy-filled (gallium and indium) strain gauge (Medasonic), with venous occlusion at 40 to 50 mm Hg for 10 of every 20 seconds. Hand blood flow was occluded by a wrist cuff inflated to 200 mm Hg. All patients came to the laboratory having fasted for 6 hours before the study. At the completion of the first part of the study, i.e., after the first time point, they were given a light nonfatty meal.

Vasodilator responses to acetylcholine (ACh, 9.25 and 37 μg/min) and sodium nitroprusside (SNP, 0.4 and 1.6 μg/min) were determined at each time point. The NO synthase inhibitor Nω-monomethyl-L-arginine (L-NMMA, 4 μmol/min) was then infused. For each drug, infusion was at 2 mL/min until the response over 3 flow measurements reached a plateau (or until a maximum of 7 minutes). Rest periods of 5 minutes between doses and drugs were observed. Normal saline (0.9% [wt/vol]) was infused during rest periods.

Spectral Analysis
Heart period variability was assessed from lead II of the ECG over a 20-minute period under resting conditions before the vascular reactivity studies. The ECG was digitized at 1000 Hz with use of a 486/50 IBM-compatible PC and a data-acquisition system incorporating a 12-bit analog-to-digital converter (McPherson Scientific). The data-acquisition system used a variable-threshold peak-detection technique from which the RR interval was derived. Heart period segments of 128-second duration were sampled at 2 Hz to create 256-point data sets. For each 20-minute recording, 16 sets of 256 points overlapping by half were processed. The linear trend was removed from each data set to eliminate its contribution to low-frequency power, and a Hanning window in the time domain was used to attenuate “spectral leakage.” Spectral analysis was performed by use of a direct fast Fourier transform. The frequency resolution was 0.0078 Hz, and the highest frequency evaluated was 0.5 Hz. The spectra obtained for different data sets were averaged to reduce variance and to sharpen reproducible spectral peaks. Power was calculated in the band range of 0.07 to 0.14 Hz (0.1 Hz or low-frequency power) and 0.14 to 0.4 Hz (respiratory or high-frequency power).

Biochemical Measurements
Cortisol measurements were performed by using an Abbott TDxFLx analyzer; cholesterol, triglyceride, and glucose levels were analyzed with a Kodak Ektachem DT 60 analyzer.

Inflammatory Markers
IL-6 measurements were performed with an Immulite IL-6 kit manufactured by Euro/DPC LTD. CRP was measured by using the revised CRP flex reagent cartridge (Dade Behring) according to a particle-enhanced turbidometric immunoassay technique.

Data Analysis
Data are presented as mean ± SEM. Summary statistics were compared by paired or unpaired Student t test where appropriate. Comparison of flows between different doses and time points was performed by 2-way repeated-measures ANOVA followed by post hoc t tests with the appropriate corrections. The level of statistical significance used was P < 0.05.

Results
Clinical profiles for CAD patients and control subjects are shown in Tables 1 and 2.

Analysis of the 2:00 PM data demonstrated no significant differences between the 2 study days for any of the variables. The 95% CIs for the within-subject differences were −67.1 to 39.9 nmol/L for cortisol, −1.56 to 1.76 mg/dL for CRP, and −0.90 to 0.11, −1.32 to 3.58, −1.13 to 0.91, and −0.48 to 0.48 mL · 100 mL−1 · min−1 for forearm blood flow at rest, after ACh, after SNP, and after L-NMMA, respectively. There was also no evidence of a significant order effect; therefore, the 2:00 PM data were averaged from the 2 study days, and the mean value was used in subsequent data presentation and analysis.
Thus, values at 8:00 AM, 2:00 PM, and 8:00 PM were 320 ng/mL, with higher values in the CAD group (7.1 vs 6.8 ng/mL at 8:00 AM; 7.1 vs 6.4 ng/mL at 2:00 PM; 7.1 vs 7.9 ng/mL at 8:00 PM; 3-way repeated-measures ANOVA, P<0.001). Similarly, the ratio of low to high frequency was highest at 8:00 AM, whereas glucose levels were significantly lower at 8:00 AM (1-way repeated-measures ANOVA, P<0.01) in both groups. There were no significant differences between control and CAD subjects in any of these biochemical parameters at each comparable time point (Table 3).

**Inflammatory Markers**

IL-6 values were below the detection limit of the assay (5 pg/mL) in all patients at all time points except in 2 CAD patients at different times (7.1 and 8.0 pg/mL). CRP was detected in all subjects but showed no significant diurnal variation. Values were higher in the CAD group than in the control group at all 3 time points, with mean values of 5.35±1.50 and 2.39±0.52 mg/dL (P<0.05), respectively. Respective values for control subjects and CAD patients were 2.78±1.04 and 4.58±1.32 mg/dL at 8:00 AM, 2.60±0.62 and 5.73±1.67 mg/dL at 2:00 PM, and 1.63±0.17 and 6.17±1.63 mg/dL (P<0.05) at 8:00 PM.

**Blood Pressure and Heart Rate**

In the control group, the resting heart rate was highest at 8:00 AM and fell later in the day (P<0.05), whereas in the CAD group, the heart rate was similar at all time points (Figure 1). In the control group, systolic blood pressure, on the other hand, was lower at 8:00 AM than later in the day (P<0.05), whereas there was no change in the CAD group. Both systolic and diastolic blood pressures were higher in the CAD group compared with the control group (Figure 1).

When expressed as a percentage of total power, there was a fall during the course of the day for the low-frequency component of RR-interval variability in the control group (P<0.05, Figure 2), with no change for the high frequency component (Figure 2). Neither showed any diurnal variation in the CAD group. Similarly, the ratio of low to high frequency for RR-interval variability was highest at 8:00 AM for the control group but showed no diurnal variation in the CAD group (P<0.05, Figure 2).

**Forearm Vascular Reactivity**

Basal forearm blood flows are as shown in Figure 3. Basal flows were significantly lower at 8:00 AM (P<0.05) than at 8:00 AM and 2:00 PM in the control group. Correspondingly, basal forearm vascular resistance was significantly higher at 8:00 PM than at 8:00 AM and 2:00 PM. In the CAD patients,
basal flows and vascular resistance were similar at all 3 time points. At 8:00 PM, basal forearm blood flow was significantly higher in the CAD group than in the control group (3.19 ± 0.59 versus 1.15 ± 0.15 mL · 100 mL⁻¹ · min⁻¹, P < 0.05). There was no difference in basal flows between the 2 groups at the other time points.

In the control group, increases in forearm blood flow to ACh infusion were significantly less at 8:00 PM than at 8:00 AM (2-way repeated-measures ANOVA followed by multiple comparisons, P < 0.05; Figure 4). There was no difference for the CAD group in the response to ACh among the 3 study times (Figure 4). The vasodilator response to ACh tended to be lower in the CAD group than in the control group at both 8:00 AM (P = 0.074) and 2:00 PM, although this was statistically not significant.

Blood flow increases to SNP were similar between control and CAD groups at all time points and showed no diurnal variation in either group (Figure 5). In the control group, L-NMMA significantly reduced forearm blood flow at both 8:00 AM and 2:00 PM but not at 8:00 PM (by paired t test). In the CAD group, there was no diurnal variation in the responses to L-NMMA (Figure 6).

**Discussion**

A diurnal variation in the presentation of cardiovascular clinical events, including acute myocardial infarction, is well established, and a number of biochemical, hemodynamic, and hematologic parameters have also been shown to undergo similar time-dependent changes. The principal findings of the present study are that endothelium-dependent vasodilation is also subject to diurnal variation and that this differs between normal male volunteers and those with established CAD. These changes may contribute to the diurnal pattern in the clinical presentation of acute coronary syndromes.

Changes in plasma cortisol concentration indicated an underlying diurnal rhythm for both asymptomatic control subjects and patients with CAD, with levels in the evening being <50% the levels of the early morning. Plasma glucose levels were also lower in the evenings. There were no differences in cortisol or glucose concentrations between normal control subjects and patients with CAD. During the day, normal volunteers also demonstrated a progressive fall in heart rate and in the proportion of low-frequency power in RR variability that was consistent with higher sympathetic activity during the morning. A diurnal variation in sympathetic
activity is well established. However, systolic blood pressure was lowest in the morning. In contrast to the normal volunteers, subjects with CAD did not show similar diurnal changes in either heart rate or low-frequency heart rate variability. Previous studies have shown that heart rate variability differs more from day to night in those without CAD compared with those with chronic stable angina.

The difference between normal subjects and those with CAD seems unlikely to be due to the effects of medication, inasmuch as β-blockers and calcium antagonists have recently been shown to have no effect on endothelial function, and the effect of ACE inhibitors is controversial. Furthermore, these medications were stopped 2 weeks before the study. However, the relatively low cholesterol in the CAD group may indicate that there were residual effects of lipid-lowering therapy in the 7 subjects previously treated. However, there were no differences between the CAD and control groups at the time of study.

Inflammatory markers, including both CRP and IL-6, have been shown to be elevated in a number of coronary syndromes and to be predictive of future events. Interestingly a relationship between such markers and endothelial function has recently been noted. In the present study, CRP levels were higher in the CAD group, in line with previous findings. IL-6 levels were undetectable in almost all subjects, which was probably attributable to the fact that CAD subjects were studied several months after their acute events. There was, as expected from the known plasma half-life (19 hours), no diurnal variation in CRP levels that could directly contribute to diurnal differences in endothelial function between CAD patients and control subjects.

Normal subjects showed a greater increase in forearm blood flow to the endothelium-dependent dilator ACh at 8:00 AM than at 8:00 PM. In contrast, there was no diurnal difference in the CAD group. The vasodilator response to ACh tended to be lower in the CAD group compared with the control group at both 8:00 AM and 2:00 PM, although this was statistically not significant. The trend is in keeping with an established association of CAD with dampened endothelium-dependent vasodilatation. Responses to the endothelium-independent dilator SNP were similar at all 3 time points, both within and between the 2 groups. It is well known that responses to nitrate donors are similar between atherosclerotic and disease-free arteries. Because basal flows were significantly lower at 8:00 PM than at the other time points in the control population, it is possible that the depressed response to ACh at this time reflects the lower basal flow.
observed in this group. However, if this were the case, the same depressed response would have been expected for SNP; this was in fact not observed.

Normal volunteers showed a reduced response to L-NMMA at 8:00 PM compared with 8:00 AM, whereas there was no diurnal variation in the responses to this drug in the CAD group. These results are consistent with an increased basal and evoked endothelial vasodilator function in the morning in normal subjects but not in patients with CAD. As discussed above, these results may be confounded by the significantly lower basal blood flow measurement at 8:00 PM in the control population.

Endothelial function was assessed in terms of vasodilatation, which was believed to be due to release from the endothelium of NO.24 The changes observed may be due to alteration in the release and/or the biological action of NO and could contribute in a number of ways to both the development of atherosclerosis and to its conversion to clinical events. These include changes in the propensity of the endothelium to permit monocyte and leukocyte attraction and penetration,6 changes in thrombosis and fibrinolysis, and changes in shifting the balance between constriction and dilatation.25 Several of these mechanisms could also influence the time of onset of clinical presentation. In the present study, information on the time of presentation was accurately available only in regard to the initial presentation at the emergency department. This occurred between 6:00 AM and 2:00 PM in 6 of the 10 subjects. Clearly, the onset of symptoms occurred earlier than the time of presentation. Therefore, these data are consistent with previous studies8 showing an \( \approx 30\% \) increase in the onset of symptoms between 6:00 AM and 12:00 noon than would be expected.

Changes in endothelium function during the course of the day could be secondary to a number of other factors. Endothelial function is sensitive to plasma cholesterol concentrations over a wide range.26 However, there was no change in cholesterol concentration during the course of the day in either group. Endothelial function is also sensitive to triglyceride-rich lipoproteins.27 Care was taken in the present study to eliminate the confounding effects of meal consumption, and there were no systematic changes in plasma triglycerides during the day. The endothelium is sensitive to shear stress, which is dependent on blood flow and viscosity, and a diurnal variation in shear could have contributed to the

Figure 5. Forearm blood flow responses to SNP in control (top) and CAD (bottom) groups. Analysis was by 2-way repeated-measures ANOVA followed by Student-Newman-Keuls test.

Figure 6. Forearm blood flow responses to L-NMMA. Analysis was by 2-way repeated-measures ANOVA followed by Student-Newman-Keuls test.
changes seen. Although plasma viscosity was not measured in the present study, several earlier studies have shown that the hematocrit falls during the course of the day, so that early morning increases in viscosity are plausible and could contribute to the increase in shear stress.28

There have been several previous studies in which basal nutritive blood flow to the limbs has been measured at various times of the day in normal volunteers as well as in specific disease groups. These studies have reported morning blood flow to be less than flow in the early evening29 for there to be no diurnal variation30 and for flow during sleep to be greater than during the awake hours.31 The reasons for the different findings are unclear but likely result from differences in subject selection, particularly regarding age and perhaps sex, as well as differences in the specific protocols followed. In addition to finding a lower morning basal flow, the study of Panza et al29 also differed from the present study in finding that the absolute flow after SNP infusion was significantly greater in the evening than in the morning.

In conclusion, the present study has demonstrated a diurnal variation in endothelium-dependent vasodilatation with enhanced activity evident in the earlier part of the day in normal subjects but not in those who had presented with acute coronary syndromes. Enhanced endothelial function during the earlier part of the day may normally act to counteract some of the potentially adverse consequences of the diurnal variation in hemodynamic parameters and in thrombosis and fibrinolysis. Further studies are required to establish whether the loss of diurnal variation in endothelial function also contributes to the development of atherosclerosis as well as to the timing of its clinical presentation.

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