Circadian variation in vascular tone and endothelial cell function in normal males

Khalid ELHERIK, Faisel KHAN, Margaret McLAREN, Gwen KENNEDY and Jill J. F. BELCH
University Department of Medicine, Ninewells Hospital and Medical School, Dundee, Scotland DD1 9SY, U.K.

ABSTRACT

The existence of circadian rhythms in the time of onset of acute cardiovascular events has been described previously. This report describes the circadian variation in endothelial cell products, such as nitric oxide (NO) and endothelin-1 (ET-1) levels, and endothelium-dependent and -independent vasodilation in normal males. Plasma ET-1 and NO were measured every 4 h in nine subjects (20–41 years old) over a 24 h period. Endothelium-dependent and -independent vascular responses were measured in the forearm skin every 4 h using laser Doppler imaging after iontophoresis of increasing doses of acetylcholine (ACh) and sodium nitroprusside respectively.

A statistically significant circadian variation was demonstrated for the mean ACh response ($P = 0.0001$, ANOVA). The peak response [in arbitrary perfusion units (AU)] occurred at 16.00 hours (8.90 ± 1.91 AU) and the lowest response at 08.00 hours (4.57 ± 0.66 AU). A significant circadian variation was also seen for the highest dose of sodium nitroprusside ($P = 0.036$, ANOVA), the peak occurred at 16.00 hours (3.97 ± 1.80 AU), and the lowest at 04.00 hours (2.62 ± 0.58 AU) and 08.00 hours (2.58 ± 1.16 AU). There was a significant circadian variation in the ET-1 levels ($P = 0.04$) with two peaks, one at 20.00 hours (0.80 ± 0.28 pg/ml) and the other at 08.00 hours (0.84 ± 0.15 pg/ml). The lowest value occurred at 16.00 hours (0.61 ± 0.24 pg/ml). There was also a borderline trend for a circadian variation in NO levels ($P = 0.06$), with higher levels at 20.00 hours (15.53 ± 8.42 μmol/l), and low levels at 04.00 hours (10.87 ± 4.70 μmol/l) and 08.00 hours (9.82 ± 3.15 μmol/l). ACh responses were significantly correlated with ET-1 ($r = -0.3, P = 0.02$) and NO ($r = 0.30, P = 0.02$) levels. Our findings suggest that endothelial activity has a circadian variation with attenuation in the morning. These circadian variations in endothelial activity might play an important role in the occurrence of acute cardiovascular events at this time, which are precipitated through the interplay between ET-1, NO and vascular function.

INTRODUCTION

The existence of circadian rhythms in the time of onset of acute cardiovascular events has been described in several epidemiological studies. Retrospective studies by Pell and D’Alonzo [1] suggested that there was a circadian variation in the onset of acute myocardial infarction (MI). The peak incidence occurred at 09.00 hours. Subsequent investigators have supported these previous results [1]. Thompson et al. [2] showed that the peak time of onset of chest pain in 1154 MI patients was between 06.00–08.00 hours. MI is approximately four times more likely to occur between 08.00–09.00 hours compared with between 00.00–01.00 hours [3]. Most episodes of myocardial ischemia occur in the morning hours, the peak incidences being reported between 07.00–13.00 hours [4] and 08.00–13.00 hours [5].

Sudden cardiac death is a relatively common cause of death in industrialized societies, accounting for 10–20% of all deaths. In 1987, Muller et al. [6] described a
circadian variation in the time reported on death certificates for 2203 out-of-hospital sudden cardiac deaths. The peak incidence occurred at 09.00–11.00 hours and the trough at 05.00 hours.

There are several physiological processes that could precipitate atherosclerotic plaque rupture and these may occur with maximal intensity in the early morning. The early morning increase in both heart rate and blood pressure [7] might induce stress on the plaque, which results in rupture. Several studies have shown an increase in platelet aggregation in the early morning [8], with peak aggregation occurring at 0.600–09.00 hours [9]. Previously, circadian variation in leucocyte activation [10] and endothelial function [11] have been shown. Maple et al. [12] showed that a circadian variation existed for both soluble intercellular adhesion molecule-1 and E-selectin. However, there is relatively little information about a potential circadian variation in other markers of endothelial cell function, such as nitric oxide (NO) and endothelin-1 (ET-1) activity and vascular tone in normal subjects. The primary objective of the present study was to investigate the possible existence of circadian variations in NO and ET-1 levels. The study also investigated the possible circadian variation in endothelial sensitivity in terms of endothelium-dependent and -independent vasodilation.

METHODS

Nine male subjects aged 28 ± 8.3 (mean ± S.D.) years old were recruited. The subjects were non-smokers, with no serious past medical history and were talking no regular medication. They abstained from all forms of medication for at least 14 days before the study. No alcohol was consumed for at least 3 days before the study, and subjects performed their normal daily activities during the study. Ethical permission for the study was obtained from the local ethical committee and written informed consent was obtained from each subject. On the day of study, subjects consumed a normal self-selected diet, but were asked to refrain from fatty foods, coffee and strenuous exercise. Volunteers were asked not to read or partake in any other activity during assessments. At each time point, blood was taken for assessment of plasma ET-1 and NO levels. The subjects adhered to the timetable detailed below: 12.00 hours, blood flow and blood sample 1; 13.00 hours, lunch; 16.00 hours, blood flow and blood sample 2; 19.00 hours, dinner; 20.00 hours, blood flow and blood sample 3; 22.00 hours, supper; 24.00 hours, blood flow and blood sample 4; 00.15 hours, go to bed; 04.00 hours, blood flow and blood sample 5; 08.00 hours, blood flow and blood sample 6; 08.20 hours, arise from bed; 08.30 hours, breakfast; 12.00 hours, blood flow and blood sample 7.

Blood samples

Venous blood was obtained from an antecubital fossa vein using a 19-gauge butterfly needle. Blood (10 ml) was added to two separate tubes: 5 ml (Sarstedt tube, containing 5 mg of EDTA per 5 ml of blood, as an anticoagulant) was used for the ET-1 assay and the other (containing 3.2% trisodium citrate as the anticoagulant) for the NO assay. Both tubes were placed immediately on ice and centrifuged at 1500 g for 15 min at 4 °C. The plasma was removed and frozen at −70 °C until further analyses.

ET-1 assay

Extracted plasma ET-1 levels were measured by enzyme immunoassay (R & D Systems, Abingdon, Oxfordshire, U.K.). The plasma samples were extracted as follows: an extraction solvent was added and the sample was then centrifuged. After centrifugation the supernatant was dried down into a pellet. The extracted samples were measured by a sandwich immunoassay technique. This involves the simultaneous reaction of the ET-1 present in the extracted sample or standard, with two antibodies directed against different epitopes of the ET-1 molecule. One antibody is coated on to the surface of the wells of a microtitre plate and the other is conjugated to horseradish peroxidase. Any ET-1 present forms a bridge between the two antibodies. After removal of unbound material by aspiration and washing, the amount of conjugate bound to the well is detected by the reaction with a substrate specific for the enzyme, which yields a coloured product that is proportional to the amount of conjugate (and thus the ET-1 in the sample). The coloured product was quantified spectrophotometrically. By analysing standards of known ET-1 concentration coincident with extracted samples and the plotting of a curve of signal versus concentration, the concentration of unknowns can then be determined.

NO assay

Nitrate/Nitrite Assay Kit (R & D Systems) was used for the NO assay. It is a two-step process. Firstly, it converts the nitrates in the sample to nitrite, utilizing nitrate reductase as an enzyme substrate, and then the total nitrite level is measured using the Greiss reaction. The Greiss reagents convert the nitrite to a deep purple azo compound, and photometric measurement of the absorbance due to the azo chromophore at 540 nm accurately determines the nitrite concentration. A standard curve is constructed in order to quantify the total NO (total nitrite and nitrate) concentration in the samples (μmol/l).

Measurement of vascular responses

Acetylcholine (ACh) (Sigma, St Louis, MO, U.S.A.), and sodium nitroprusside (SNP) were each made up to a 1%
solution in deionized sterile water. SNP solutions were covered with aluminium foil to avoid light exposure. Experiments were performed in a quiet, temperature-controlled laboratory set at 22–23 °C. Subjects underwent a 30 min equilibration period. The subjects were seated comfortably with the arms supported at heart level. The skin of the volar surface of the forearm was cleaned gently with one-sided tape to remove dead skin, and cleaned once again with alcohol and finally with sterile water. A direct iontophoresis electrode chamber was attached approximately midway between the wrist and elbow. Solutions (approx. 2.5 ml) were added to the chamber and were delivered transdermally by iontophoresis. The indifferent electrode was a Velcro strap soaked in deionized sterile water and placed around the subject’s wrist to complete the circuit. The leads from the electrodes were connected to a battery-powered iontophoresis controller MIC 1 (Moor Instruments, Axminster, Devon, U.K.), which provided a direct current for the delivery of solutions. ACh was iontophoresed for 10 s using an anodal current of 0.1 mA to give a charge of 1 mC. Cutaneous vascular responses were measured following iontophoresis of ACh for 100 s. To increase the dose, ACh was iontophoresed for 20 s, 40 s and 80 s (to give 2 mC, 4 mC and 8 mC charges respectively), with skin perfusion recorded for 100 s between each dose.

A 0.1 mA cathodal current was used to iontophoreses SNP for 10 s, 20 s, 40 s and 80 s to give charges of 2 mC, 4 mC and 8 mC respectively. Cutaneous vascular responses were measured for 240 s between each dose.

Skin perfusion was measured at the volar surface of the forearm using a laser Doppler perfusion imager (LDI; Moor Instruments). Moving blood in the microvasculature causes a Doppler shift, which is processed to build up a colour-coded image of blood flow. The scanning configuration covered an area of 25 cm², using 256 pixels x 256 pixels. The scan speed was 4 ms/pixel. The scanner head was set at 50 cm from the surface of the forearm. The scans were analysed using the LDI software package (Moor Instruments). Blood flow was quantified in arbitrary perfusion units (AU).

**Statistical analysis**

Blood flow values are expressed in AU as the ratio of skin perfusion after each dose of ACh and SNP divided by the baseline measurement. Data were analysed using the SPSS statistical package (version 8). As the data were not normally distributed, analysis of variance was performed using non-parametric testing. Post-hoc tests were performed to show the significant variation between the time points. Correlations were performed using the Spearman Rank correlation. All values are expressed as the mean ± S.D., unless stated otherwise. The null hypothesis was rejected at $P < 0.05$.

**RESULTS**

**Circadian variation for endothelium-dependent vasodilation**

A statistically significant circadian variation was demonstrated for endothelium-dependent vasodilation at all 4 doses of ACh ($P = 0.0001$, ANOVA). The highest value for endothelium-dependent vasodilation, based on the average 4 doses of ACh responses, occurred at 16.00 hours (5.95 ± 1.73 AU) and the lowest values occurred at the two time points, 04.00 hours (3.33 ± 0.77 AU) and 08.00 hours (3.16 ± 0.49 AU) (Figure 1).

**Circadian variation for endothelium-independent vasodilation**

There was no significant circadian variation for the mean (averaged over the 4 doses) endothelium-independent response. However, a statistically significant circadian variation was demonstrated for the highest SNP dose.
Circadian variation in ET-1 and NO levels

There was a significant circadian variation in ET-1 levels ($P = 0.04$, ANOVA). Two peaks occurred, the first at 20.00 hours ($0.80 \pm 0.28$ pg/ml) and the second at 08.00 hours ($0.84 \pm 0.15$ pg/ml) (Figure 3). The lowest value occurred at 16.00 hours ($0.61 \pm 0.24$ pg/ml).

There was a borderline trend for a circadian variation in NO levels ($P = 0.06$) with higher levels at 20.00 hours ($15.53 \pm 8.42$ μmol/l) and low levels at 04.00 hours ($10.87 \pm 4.70$ μmol/l) and 08.00 hours ($9.82 \pm 3.15$ μmol/l) (Figure 4).

Correlation of skin perfusion with plasma ET-1 and NO levels

All data points were used for the correlations, i.e. nine subjects each at seven time points. ET-1 levels were significantly correlated with the mean ACh response ($r = -0.3$, $P = 0.02$) (Figure 5). Furthermore, at the lowest response time point (08.00 hours), ACh was negatively correlated with the ET-1 level ($r = -0.8$, $P = 0.01$).

ACh responses correlated positively with plasma NO levels ($r = 0.30$, $P = 0.02$) (Figure 6). No correlations were found between SNP responses and ET-1 and NO levels ($P = 0.7$ and $P = 0.44$ respectively).

DISCUSSION

The results of the present study demonstrate a significant circadian variation in endothelium-dependent and -independent vasodilation, with maximum vasodilation responses occurring at 16.00 hours and minimum responses at 04.00 hours. It is now accepted that over 90% of MIs result from thrombotic occlusion of coronary arteries [13]. Factors that promote thrombosis are more prevalent in the morning and this is believed to contribute to the increased morning incidence in the onset of MI and other thrombotic events. Identification of the factors responsible for the circadian variation in the onset of
thrombotic events could be important clinically, as it might allow preventive therapeutic intervention at the time of maximal risk or advances in therapeutic management.

Other studies have shown circadian variations with a significant morning increase in white blood cell aggregation and free radical status [11,15], platelet aggregation [9], blood pressure and heart rate [16], cortisol and catecholamines [17]. Although reduced fibrinolytic activity has been reported [18], little attention has been paid to the possibility of a circadian variation in endothelial cell function and production of such substances as ET-1 and NO to examine their potential contribution to the increased morning incidence in onset of thrombotic events. Maeda et al. [19] studied the circadian variation of ET-1 in systemic sclerosis and in healthy females. Plasma ET-1 levels were significantly higher in systemic sclerosis throughout the day and night, but there was no significant circadian variation detected in either females with systemic sclerosis or healthy females. However, in the present study, we demonstrated a significant circadian variation in plasma ET-1 levels in normal males. These conflicting results might be due to differences in sex, age groups and study design.

Endothelium-dependent vasodilation was significantly decreased at 08.00 hours, and might be related to higher levels of ET-1 at this time point on the basis of a significant negative correlation between vascular responses and ET-1 levels. The maximum endothelium-dependent and -independent vasodilation occurred at 16.00 hours, and correspondingly at this time point, the lowest plasma ET-1 level was recorded. These findings suggest a relationship between ET-1, a potent vasoconstrictor peptide, and the reduction of vasodilation in the morning, which might contribute to the circadian variation of cardiovascular events. Some investigators have found an additional second peak of acute MI in the evening [20]. These findings could be explained by the occurrence of the second peak of ET-1 as shown in the present study.

In the present study, we measured NO levels by using a Nitrate/Nitrite assay kit in which nitrates are converted to nitrite and then the total nitrite level is measured using the Greiss reaction. However, Lauer et al. [21] have shown recently that plasma nitrite accurately reflects acute changes in endothelial NO synthase, and that this might be a more sensitive marker of endothelial NO synthase activity. Nevertheless, using our method, we did find a borderline trend for a circadian variation in NO levels with higher levels at 20.00 hours and lower levels at 04.00 hours and 08.00 hours. The minimum endothelium-dependent and -independent vasodilation occurred at 04.00–08.00 hours. Correspondingly, at these time points, the lowest plasma NO level was recorded. These findings suggest that the early morning diminution of vascular responses might also be related to a decrease in NO production or activity, combined with an increase in endothelial-derived vasoconstrictors.

These results are in keeping with the observations of Etsuda et al. [22] who examined the circadian variation of endothelium-dependent flow-mediated dilation in healthy men aged 25–32 years at four different times over the course of a day. Flow mediated dilation at 08.00 hours and 12.00 hours was significantly lower than that at 17.00 hours.

Impaired smooth muscle responsiveness to NO stimulation, impaired L-arginine availability or utilization, endothelial release of vasoconstrictor prostanoids, increased NO degradation and reduced NO synthase activity may all be implicated in this impaired response. Endothelium-derived NO can modulate the production and actions of ET-1 [23]. In the short term, NO inhibits production of ET-1 [24], whereas chronic exposure causes up-regulation of ET-1 receptors [25]. These interactions indicate the existence of a complex relationship between the endothelin and NO systems.

The recognition of the role of various factors in the circadian variation in endothelial function might result in the development of preventive strategies and/or advances in therapeutic management. Emphasis can be placed on pharmacological protection with drug administration time altered to provide protection at the time of maximum risk. In doing so, medication-free periods might be determined more precisely. Furthermore, targeting therapy to risk periods might attenuate the need for multiple drug dosing and/or attenuate side effects. The results of the current study show that endothelial function has a circadian variation and vasodilation is significantly attenuated in the morning. This morning decrease in endothelial function should be recognized in clinical research. Consequently, measurements of blood flow and endothelial function should preferably be performed at the same time of the day. Furthermore, a circadian variation in endothelial function might play an important role in the occurrence of acute cardiovascular events.

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