

Impact of the human circadian system, exercise, and their interaction on cardiovascular function

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The risk of adverse cardiovascular events peaks in the morning ($\approx 9:00$ AM) with a secondary peak in the evening ($\approx 8:00$ PM) and a trough at night. This pattern is generally believed to be caused by the day/night distribution of behavioral triggers, but it is unknown whether the endogenous circadian system contributes to these daily fluctuations. Thus, we tested the hypotheses that the circadian system modulates autonomic, hemodynamic, and hemostatic risk markers at rest, and that behavioral stressors have different effects when they occur at different internal circadian phases. Twelve healthy adults were each studied in a 240-h forced desynchrony protocol in dim light while standardized rest and exercise periods were uniformly distributed across the circadian cycle. At rest, there were large circadian variations in plasma cortisol (peak-to-trough $\approx 85\%$ of mean, peaking at a circadian phase corresponding to $\approx 9:00$ AM) and in circulating catecholamines (epinephrine, $\approx 70\%$; norepinephrine, $\approx 35\%$, peaking during the biological day). At $\approx 8:00$ PM, there was a circadian peak in blood pressure and a trough in cardiac vagal modulation. Sympathetic variables were consistently lowest and vagal markers highest during the biological night. We detected no simple circadian effect on hemostasis, although platelet aggregability had two peaks: at \approx noon and $\approx 11:00$ PM. There was circadian modulation of the cardiovascular reactivity to exercise, with greatest vagal withdrawal at $\approx 9:00$ AM and peaks in catecholamine reactivity at $\approx 9:00$ AM and $\approx 9:00$ PM. Thus, the circadian system modulates numerous cardiovascular risk markers at rest as well as their reactivity to exercise, with resultant profiles that could potentially contribute to the day/night pattern of adverse cardiovascular events.

cardiovascular disease

Cardiovascular disease is the leading cause of mortality in both men and women in developed countries (1, 2). Epidemiological studies show a clear day/night pattern in risk for myocardial infarction (3–6), stroke (7), angina (5), ventricular arrhythmias (8), and sudden cardiac death (9) with primary peaks in the morning ($\approx 6:00$ AM–noon), secondary peaks in the evening ($\approx 6:00$ – $10:00$ PM), and lowest vulnerability during the night (\approx midnight– $6:00$ AM) (reviewed in ref. 10). The morning peaks in cardiovascular events cannot be fully explained by the day/night pattern in behavioral triggers such as activity (11). The circadian system, including the suprachiasmatic nucleus (SCN) in the hypothalamus and circadian oscillators in peripheral tissues, orchestrates endogenous circadian rhythms in physiology and behavior (12). Findings from animal work show that the SCN may influence the cardiovascular system via humoral factors and multisynaptic neural projections to the heart, adrenal cortex, adrenal medulla, kidneys, and vasculature (13) and that circadian clock mutations (*Clock*, *Bmal1*, *Npas2*) lead to abnormalities in sympathetic activity, vasculature, and blood pressure stress responses (14, 15). To assess the influence of the circadian system in humans, we used the forced desynchrony (FD) protocol, the gold standard to dissociate the separate influences of the circadian system and behavioral/environmental influences upon variables of interest (16). In addition, by scheduling individuals to live on non-24-h days, the FD protocol enabled the comparison of

the effects of standardized behavioral stressors occurring at different phases of the circadian cycle. Based on these principles, we tested two hypotheses: (i) the circadian system modulates autonomic, hemodynamic, and hemostatic biomarkers at rest; and (ii) reactivity of these biomarkers to exercise is altered at different circadian phases. We sought to determine whether circadian rhythms in specific markers of cardiovascular risk match the day/night pattern in adverse cardiovascular events (primary morning peak, secondary evening peak, and overnight trough).

Results

Each of 12 healthy subjects was scheduled to live on recurring 20-h “days” for 240 h (FD protocol) in an individual laboratory suite while we repeatedly assessed a cardiovascular biomarker panel during rest and standardized exercise (Fig. 1). This approach enabled us to quantify the independent influences of the circadian system (endogenous cycle length close to 24 h) and behaviors (imposed cycle length of 20 h), and their interacting effects on the cardiovascular biomarker panel.

Circadian Phase and Period. In the 2-wk period before entering the laboratory, average habitual waketime was $7:38$ AM ± 15 min. In the laboratory, circadian period was 24.09 ± 0.06 h (mean \pm SEM; range: 23.81 – 24.63 h), similar to prior reports (16, 17), and core body temperature minimum (CBTmin) occurred at $4:33$ AM ± 19 min (3.08 ± 0.26 h before habitual waketime). For each individual, CBT was used as the circadian phase marker and the time of CBTmin was assigned as 0 circadian degrees, with one full circadian cycle spanning 360 circadian degrees. For clarity, when describing circadian time we also refer to the mean approximate corresponding time of day during normal entrainment in these subjects. Thus, 0° was $\approx 4:30$ AM, 60° was $\approx 8:30$ AM, and 120° was $\approx 12:30$ PM, etc. in these subjects.

For each variable, we tested for the existence of circadian rhythmicity at rest (Hypothesis 1) and in reactivity to exercise (Hypothesis 2). We fitted the data to test each hypothesis by using cosinor analyses that included both a fundamental circadian cycle (≈ 24 h) and a cycle with twice the frequency (second harmonic; ≈ 12 h) (Fig. S1). Circadian rhythms in cardiovascular variables are described below by their peak-to-trough rhythm amplitudes and the phases at which their peaks and/or troughs occur. Our specific prediction was that cardiovascular risk

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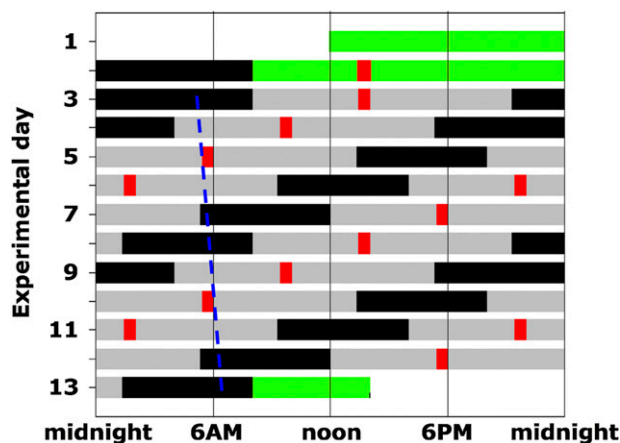


Fig. 1. Forced Desynchrony Protocol. Solid black bars, scheduled sleep (0 lux); gray bars, wakefulness in dim light (≈ 1.8 lux (≈ 0.0048 W/m²)); green bars, baseline days and discharge day in normal room light (≈ 90 lux); red bars, test batteries; dashed blue line, illustration of trajectory of circadian CBTmin throughout the FD protocol (circadian period of 24.09 ± 0.06 h in these subjects).

markers would have circadian peaks (or troughs for ‘protective’ cardiac vagal markers) during rest and/or in response to exercise at times that would correspond to the most vulnerable window for adverse cardiovascular events ($\approx 6:00$ AM–noon).

Autonomic Nervous System and Cortisol. At rest, both plasma epinephrine and norepinephrine had large and significant circadian variations with average amplitudes of 70% ($P < 0.0001$) and 34% of the mean ($P = 0.0009$), respectively (Fig. 2, *Left Top*, black lines; Table 1 and Fig. S2). Both epinephrine and norepinephrine had troughs during the biological night and peaks across the biological day, without clear peaks in the primary vulnerable phase ($\approx 9:00$ AM, 70°). The circadian effect on catecholamine levels was large, being almost the same in magnitude as the effect of the standardized exercise protocol designed to sustain 60% of maximum heart rate (+88 and 67%, respectively; Fig. 2, difference between black and red lines). In fact—considering the underlying circadian rhythmicity—when subjects were at rest around midday, surprisingly, their epinephrine levels were as high as those during exercise at $\approx 4:30$ AM (Fig. 2, *Left Top*).

In addition, the reactivity of epinephrine and norepinephrine to exercise varied significantly across the circadian cycle. However, rather than a simple ≈ 24 -h rhythm, this effect was characterized by a ≈ 12 -h rhythm with two large peaks at ≈ 7 – $10:30$ AM (40 – 90°) and $\approx 8:00$ – $10:00$ PM (230 – 260°) for both epinephrine and norepinephrine ($P = 0.039$ and $P = 0.011$, respectively; Table 2 and Fig. S3). These 12-h rhythms in reactivity to exercise were similar to the 12-h component of the rhythms of the resting data (Fig. S1). Notably, there was a 2-fold larger exercise-induced increase in epinephrine at $\approx 8:30$ AM (60° ; $+84 \pm 15\%$; Fig. 2, *Left Top Upper*) compared with the least vulnerable period, i.e., the middle of the night ($\approx 4:30$ AM, 0° ; $+44 \pm 11\%$) ($P = 0.01$, Student’s t test). This difference was the largest among any two consecutive time windows, identifying 8:30 AM as a time of exaggerated physiological transition.

At rest, both frequency and time indices of cardiac vagal tone (HF power and pNN50, respectively; see *Methods*) had significant circadian rhythms. The amplitude was 34% for HF power ($P = 0.003$) and 21% for pNN50 ($P = 0.001$) (Fig. 2, *Left Bottom*; Table 1 and Fig. S2). These “protective” cardiac vagal markers had peaks at $\approx 7:00$ – $8:00$ AM (40 – 50°) and troughs around the time of the secondary peak in cardiovascular vulnerability ($\approx 6:00$ – $10:30$ PM, 200 – 270°).

As hypothesized (Hypothesis 2), there were significant circadian rhythms in the reactivity of vagal markers to exercise (35% and 21% for HF and pNN50, respectively), with the greatest vagal withdrawal in the vulnerable ≈ 7 – $8:30$ AM period (40 – 60°) (Table 2 and Fig. S3).

At rest, plasma cortisol had a pronounced circadian rhythm ($P < 0.0001$), with an amplitude of 85%, a sharp peak at $\approx 8:30$ AM (60°) and a trough in the biological evening ($\approx 10:30$ PM, 270°) (Fig. 2, *Left Middle*; Table 1 and Fig. S2). We could not reliably analyze the effect of exercise on cortisol because exercise bouts were 15 min, whereas cortisol peaks ≈ 30 min after the start of exercise (18).

Hemodynamics. At rest, both systolic blood pressure (SBP) and diastolic blood pressure (DBP) had significant circadian variations, with amplitudes of 5.5% and 4.1%, respectively (both $P < 0.0001$). Contrary to expectations, the peaks in SBP and DBP occurred at the end of the biological day ($\approx 6:00$ – $8:00$ PM, 200 – 230°), the secondary vulnerable phase, rather than at the primary vulnerable phase of $\approx 8:30$ AM (60°) (Fig. 2, *Right Top*; Table 1 and Fig. S2).

Exercise significantly increased SBP by 37.9 mmHg ($P < 0.0001$) and DBP by 7.6 mmHg ($P = 0.0017$). In addition, the reactivity of SBP to exercise, but not DBP, had a significant circadian rhythm (7.2 mmHg; 6.4%; Table 2 and Fig. S3). Again, contrary to expectations, the smallest SBP reactivity occurred around the time of the primary vulnerable phase ($\approx 10:00$ AM, 80°) and the greatest SBP reactivity occurred at the beginning of the biological night (\approx midnight, 300°).

At rest, heart rate (HR) had significant circadian variation, with an amplitude of 8.6% ($P < 0.0001$), a peak during the middle of the biological day ($\approx 4:00$ PM, 200°) and a trough during the biological night ($\approx 4:00$ AM, 350°) (Fig. 2, *Right Middle*; Table 1 and Fig. S2). There was a significant increase in HR with exercise of 54.6 bpm ($P < 0.0001$), but there was no significant circadian rhythm in HR reactivity to exercise.

Hemostasis. At rest, platelet aggregability had significant circadian variation, with an amplitude of 12% ($P = 0.04$), similar in magnitude to the effect of exercise (see below). Rather than a simple ≈ 24 -h rhythm, platelet aggregability had a ≈ 12 -h rhythm with peaks at both \approx noon (110°) and $\approx 11:00$ PM (280°) (Fig. 2; Table 1 and Fig. S2). Exercise significantly increased platelet aggregability by 9% ($P < 0.0001$) without a significant circadian effect.

At rest, platelet count had no significant circadian variation (Fig. 2; Table 1 and Fig. S2). Exercise increased platelet count by 10% ($P < 0.0072$) but without significant circadian variation in reactivity to exercise.

Discussion

We found that, independent of environmental and behavioral changes, the endogenous circadian system modulates many components of cardiovascular control in humans at rest including sympathetic activity, cortisol, cardiac vagal modulation, HR, arterial blood pressure (≈ 24 -h rhythms), and platelet aggregability (harmonic ≈ 12 -h rhythm). Remarkably, the circadian system also modulated the vagal, sympathetic, and SBP reactivity to exercise.

Potential Mechanisms Involved in Circadian Variation in Cardiovascular Biomarkers. The potential underlying mechanisms of the circadian variation in cardiovascular biomarkers may include humoral circadian rhythms driven by the SCN (e.g., ACTH), multisynaptic neural projections from the SCN to the organs (e.g., heart, vasculature, and adrenal gland), and/or peripheral oscillators located in these organs (13, 19, 20). Circadian rhythms in autonomic nervous system activity and humoral factors, in turn, could contribute to synchronization of peripheral oscillators in

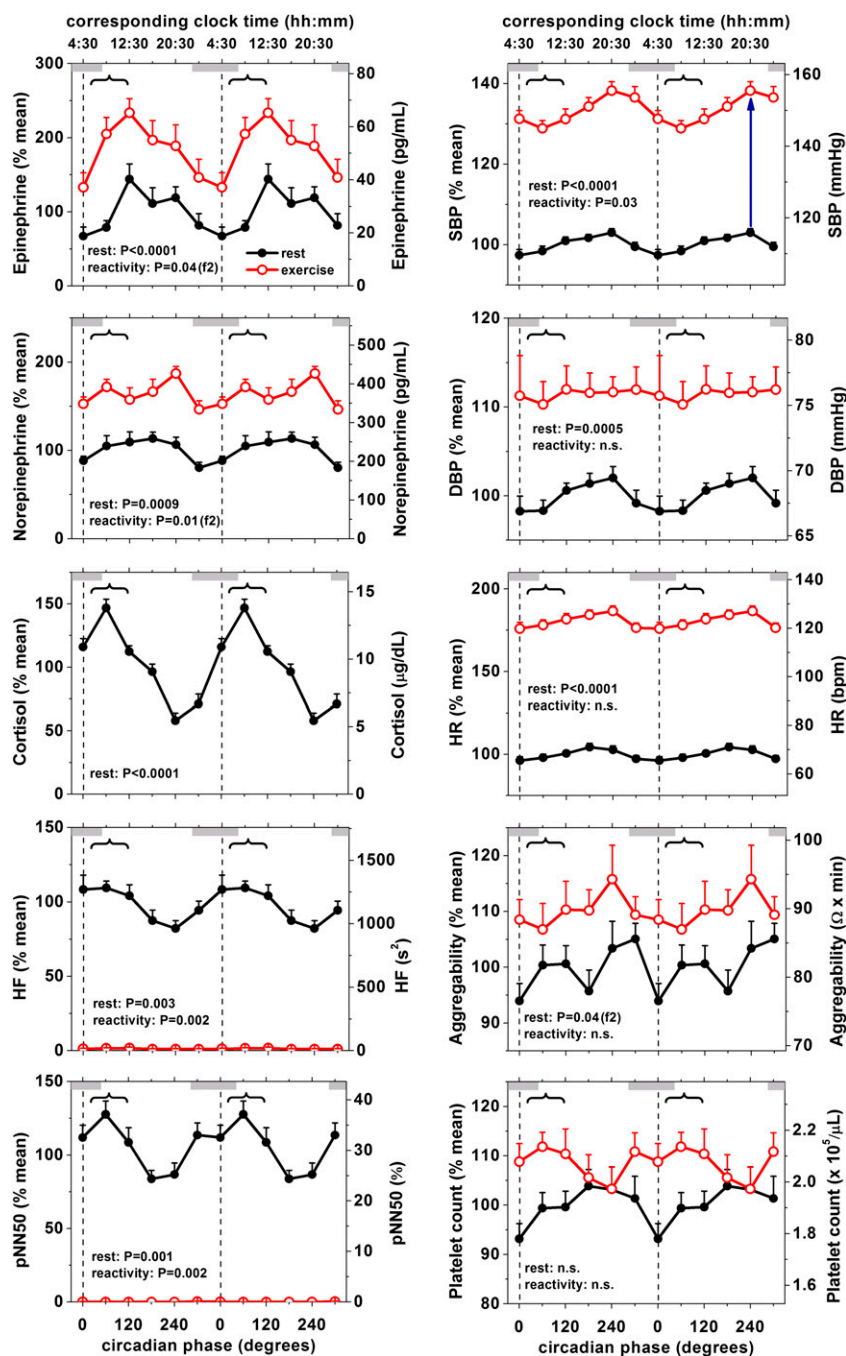


Fig. 2. Circadian rhythm in cardiovascular function at rest and during exercise. There were significant endogenous circadian rhythms at rest for plasma epinephrine, norepinephrine, cortisol, HF, pNN50, SBP, DBP, and HR. Platelet aggregability had two peaks across the circadian cycle. Platelet count had no significant circadian variation. Influence of exercise on cortisol could not be assessed reliably (see text) and is not displayed. The influence of the circadian system on reactivity to exercise is shown in Fig. S3. Data are expressed as a percentage of each individual's rest values across the FD protocol (left y axis) in addition to the absolute values (right y axis). Black circles, rest; red circles, exercise; error bars, SEM; gray bars, group average habitual sleep episodes; vertical dotted lines, CBTmin; curly brackets, most vulnerable period for adverse cardiovascular events observed in epidemiologic studies (≈6:00 AM–noon); *P* values, significance of circadian effect on resting values (rest) and in response to standardized exercise (reactivity) assessed by cosinor model; f2 (after *P* values), significance of second harmonic of circadian rhythm; blue upward arrow in *Right Top Upper*, example of reactivity to exercise at one particular circadian phase as analyzed in Fig. S3.

the heart and vasculature (21, 22). Surprisingly, we also found evidence for the existence of endogenous ≈12-h rhythm in cardiovascular biomarkers, specifically in platelet aggregability and in the reactivity to exercise of epinephrine and norepinephrine. Such 12-h patterns could be related to 12-h rhythms as have been demonstrated in animal studies, e.g., cardiac β -adrenergic receptor density and function (23), hormonal rhythms with a shap-

ing role for the SCN (24), and gene transcription in various tissues, including liver, heart, kidney, and adrenals (25). Endogenous 12-h rhythms may have specific relevance to anticipating the daily transitions of dusk and dawn (25).

Comparison with Previous Studies. There are clear day/night patterns of catecholamine release and in blood pressure in humans

Table 1. Circadian variation during rest

Variable	<i>P</i> value circadian	<i>P</i> value harmonic	Timing trough, °	Timing peak, °	Amplitude	Amplitude, %
Epinephrine	<0.0001	n.s.	0	220	19.8 pg/mL	70.3
Norepinephrine	0.0009	n.s.	330	190	77.0 pg/mL	33.6
Cortisol	<0.0001	n.s.	270	60	7.91 µg/dL	84.9
HF	0.0028	n.s.	270	50	394 s ²	34.1
pNN50	0.0011	n.s.	200	40	6.08%	20.9
SBP	<0.0001	n.s.	10	230	6.16 mmHg	5.5
DBP	0.0005	n.s.	30	200	2.78 mmHg	4.1
HR	<0.0001	n.s.	350	200	5.87 bpm	8.6
Aggregability	n.s.	0.043	20; 190	110; 280	10.01 Ωxmin	12.3

Platelet count was not significant and thus not shown. n.s., not significant.

(10). However, few studies have standardized behavioral and environmental factors across the day/night cycle, which is a requirement to separate endogenous circadian from behavioral/environmental effects (e.g., induced by the daily rest/activity cycle). Linsell et al. (26) had indirect evidence of a circadian rhythm in plasma epinephrine (but not norepinephrine) based on 24-h fluctuations in epinephrine that could not be fully explained by sleep and/or posture. No daily rhythm in plasma or urinary epinephrine was found in a study in which subjects remained supine, but sleep/wake, feeding, and lighting conditions were not strictly controlled (27). However, in a previous study using well-established circadian techniques, i.e., the FD protocol, we observed an endogenous circadian rhythm in urinary epinephrine, but not in urinary norepinephrine, with a circadian peak at ≈4:00 PM (17), similar to the midpoint of the broad peak of plasma epinephrine in the current study (Fig. S1 and Fig. S2). Using plasma assessment of catecholamines, the current study confirms the circadian rhythm of epinephrine and identifies a circadian rhythm in norepinephrine. The circadian rhythm in plasma norepinephrine reflects whole body spillover of norepinephrine from sympathetic nerves and adrenal medulla into the circulation, which is likely a more important measure of sympathetic influence on the heart and vasculature than urinary norepinephrine that is affected more by kidney function (28).

Our finding of circadian rhythms in SBP and DBP contrasts with two studies that found no circadian rhythms in BP (29, 30). These previous studies might have suffered from some methodological limitations because their subjects: (i) were exposed to a relatively high luminance (up to 100 lux) that can greatly affect the circadian system (31); (ii) were permitted “bathroom breaks” that interrupted their semirecumbent posture; and (iii) had their BP data aligned based on habitual sleep times rather than a much more accurate circadian phase marker, such as CBTmin. Moreover, we recently confirmed the endogenous circadian rhythmicity of arterial blood pressure in humans in two additional complementary protocols (FD and constant routine protocols) (32).

The current findings of circadian rhythms in cardiac vagal markers (peaking during the biological night and morning), HR (biological day) and cortisol (≈9:00 AM) are consistent with other valid circadian studies that use constant routine or FD pro-

ocols (ref. 17; for review, see ref. 33). The circadian influence on platelet aggregability is consistent with animal experiments, showing effects of core molecular clock components on coagulation (34).

The circadian rhythm in reactivity of SBP and epinephrine in response to exercise is reminiscent of the daily rhythm in reactivity in response to restraint stress in mice that is circadian clock gene-dependent (*Bmal1*) (14). Furthermore, recent animal experimental data indicate increased susceptibility to cardiac ischemia/reperfusion at the sleep-wake transition that is mediated by the cardiomyocyte circadian clock, providing further support for the involvement of the circadian system in the timing of adverse cardiovascular events (35).

Potential Implications for Day/Night Pattern of Adverse Cardiovascular Events. This study in healthy humans provides a unique dataset on circadian amplitude and phase of multiple cardiovascular biomarkers, both at rest and in the reactivity to a behavioral stressor. These data have possible implications for the day/night pattern of adverse cardiovascular events and provide a basis for comparison with studies of more vulnerable populations. For instance, the magnitude of circadian influences on many cardiovascular biomarkers at rest was considerable, with peak-to-trough differences of ≈35–85% for epinephrine, norepinephrine, cortisol, and cardiac vagal modulation. Superimposed on these observed rhythms during the resting state, there were large changes across the circadian cycle in physiological reactivity to exercise, in the range of ≈35–50% for our autonomic markers. Even the smaller circadian modulation of SBP of 6 mmHg (5%) at rest may be of clinical relevance, because this order of magnitude is comparable to that achieved by antihypertensive medications (36). Moreover, we found an additional 7 mmHg (6%) circadian rhythmicity in SBP reactivity to mild exercise that is superimposed on the underlying circadian SBP rhythm.

Even at rest, there are a number of risk factors that had their greatest potential circadian rhythm-related effects at the specific phase corresponding to the morning peak in adverse cardiovascular events (≈6:00 AM–noon), including the circadian peak in plasma cortisol, the first 12-h peak of platelet aggregability, and the greatest rise (i.e., rate of change, not peak) of epinephrine (Fig. 2

Table 2. Circadian variation of the reactivity to the exercise stressor

Variable	<i>P</i> value circadian	<i>P</i> value harmonic	Timing minimum response, °	Timing maximum response, °	Amplitude	Amplitude, %
Epinephrine	n.s.	0.039	180; 350	90; 260	14.7 pg/mL	52.1
Norepinephrine	n.s.	0.011	130; 320	40; 230	79.5 pg/mL	34.6
HF	0.0023	n.s.	270	60	405 s ²	35.1
pNN50	0.0015	n.s.	200	40	6.06%	20.8
SBP	0.034	n.s.	80	300	7.23 mmHg	6.4

DBP, HR, aggregability, and platelet count were not significant and thus not shown; cortisol not shown, see text. n.s., not significant.

and Fig. S2). There are daily rhythms in sensitivity to catecholamines in different organ systems including the heart, due in part to the down-regulation of adrenergic receptors after stimulation (23, 37–39). The circadian-mediated rapid rise in epinephrine that we observed at $\approx 8:00$ AM may be particularly problematic in the setting of the concomitant circadian peak in cortisol because cortisol has permissive effects on adrenergic receptors (40). Based on epinephrine's effects on blood clotting (41), myocardial oxygen demand, and vasoconstriction (42), we would speculate that the circadian epinephrine rhythm may contribute to the morning peak in adverse cardiovascular events (3–10).

In contrast, the peak in vagal cardiac markers and the trough in SBP and DBP at circadian phases corresponding to $\approx 6:00$ AM–noon in these healthy control subjects may be protective (36, 43), possibly counterbalancing factors promoting cardiovascular risk at that circadian time. In contrast, because hypovagotonia is common in populations at risk for adverse cardiovascular events (44), the protective circadian peak in vagal tone in the morning may be reduced in patients who manifest the morning peak in cardiovascular risk. Of note, at circadian phases corresponding to the vulnerable time of $\approx 6:00$ AM–noon, we observed relative coactivation of sympathetic and vagal activity. Sympathovagal coactivation may maximize precise control of end organ responses, increase cardiac output, but also increase risk for arrhythmia in vulnerable populations (45). Further studies are required to test the pathophysiological consequences of circadian influences on autonomic coactivation. Furthermore, loss of circadian rhythmic variation in vagal tone may tip the balance toward morning events during exercise.

Interestingly, the secondary peak in adverse cardiovascular events in the early evening ($\approx 6:00$ – $10:00$ PM) coincides with, and therefore might be explained by, the peaks of norepinephrine, HR, SBP, and DBP, the second harmonic peak of platelet aggregability, and the second harmonic peak in reactivity of epinephrine and norepinephrine, as well as the trough in cardiac vagal modulation at a circadian phase corresponding to $\approx 5:00$ – $10:30$ PM.

As we hypothesized, the endogenous circadian trough in most of the cardiovascular risk biomarkers, as well as the circadian peak in “protective” vagal markers, occurred during the biological night (\approx midnight– $8:00$ AM; $\approx 300^\circ$ – 50°) for most variables (except for cortisol: $10:30$ PM; 270° ; Table 1), possibly underlying the nighttime trough in adverse cardiovascular events in epidemiological studies (3–10).

Regarding the circadian effect on reactivity to exercise, the phase at which exercise resulted in the maximum vagal withdrawal and the first harmonic (≈ 12 -h) peaks for epinephrine and norepinephrine corresponded to the most vulnerable time of day ($6:00$ AM–noon), with the epinephrine response to exercise doubling from $\approx 4:30$ AM (0°) to $\approx 8:30$ AM (60°). Thus, such a circadian rhythm in sympathovagal reactivity to behavioral stress could also contribute to the morning peak in adverse cardiovascular events (Fig. S4).

Integration and Summary. Knowledge about circadian influences on mechanisms involved in cardiovascular risk is important for optimizing the timing of therapy, preventing behavioral triggers at potentially vulnerable circadian phases, and selecting the timing of diagnostic assessments. Furthermore, the interaction between the circadian system and behavioral influences may be especially important for the millions of night workers, jet travelers, and those affected by sleep disorders, in whom the temporal coupling between the endogenous circadian timing system and behavioral stressors is disturbed (46).

As expected from the complex physiology, no single variable exhibited a circadian profile that fully explained the timing of adverse cardiovascular events seen in population studies. Combinations of risk factors possibly summate to produce the day/night

pattern in adverse cardiovascular events, as proposed in our simplified conceptual model (Fig. S4). Finally, the daily distribution of triggering behaviors may be the most important factor, although our data point to the fact that interactions between the circadian system and reactivity to behaviors need to be considered.

Methods

Subjects. Twelve (6 male; 6 female) consenting healthy volunteers [mean \pm SD (range); 25.8 ± 5.7 y (20–42 y); BMI: 23.6 ± 3.2 kg/m² (19.9–29.6 kg/m²)] completed a 13-d inpatient local Human Research Committee-approved protocol at our Clinical and Translational Research Center. Subjects were healthy as confirmed by extensive history, physical, psychological, and laboratory examination. Subjects maintained a regular sleep/wake cycle of 8 h sleep per night for 2–3 wks before admission (verified by sleep/wake diaries, call-in times to a time-stamped voice recorder and wrist actigraphy). Subjects reported no shift work for 3 y and not crossing more than one time zone for 3 mo before the study. To minimize training and detraining effects while in the study, subjects maintained an exercise level equivalent to a 30-min brisk walk per day for the 2–3 wks immediately before admission. Toxicology screens upon admission confirmed that subjects were free of any drugs, including caffeine, alcohol, and nicotine.

Study Protocol. Subjects spent 13 d in a private laboratory room free of time cues, including two baseline days and nights (16 h scheduled wakefulness and 8 h scheduled sleep) followed by a “forced desynchrony” protocol (FD) consisting of 12 20-h “days” (13:20 hours scheduled wakefulness; 6:40 hours scheduled sleep; maintaining a 1:2 sleep:wake ratio) in dim light (≈ 1.8 lux at the horizontal angle of gaze) to minimize influences on the circadian system (Fig. 1). Meals were given at 1:25 hours (breakfast), 6:45 hours (lunch), 10:45 hours (dinner) and 12:10 hours (snack) after scheduled waketime. Because the 20-h rest-activity cycle in dim light is outside the range of entrainment, the circadian timing system runs free at its inherent rate of close to 24 h (16). Thus, the sleep and wake episodes, and the exercise tests, were uniformly distributed across the circadian cycle. The exercise test was scheduled to start 5:25 hours after scheduled awakening, consisting of 25 min rest while seated on an ergometer and 15 min cycling on an ergometer (Cybex Semi-recumbent Cycle Ergometer) at 60% maximum HR (see *SI Methods* for details). During each exercise test session, subjects maintained the same body posture (seated), and were asked to limit any unscheduled movement and not to talk. As opposed to in experimental animals, exercise appears to be a weak *Zeitgeber* for the circadian system in humans and does not affect circadian period (47). Indeed, the observed circadian period in the current study (average 24.09 h) was remarkably similar to that reported in previous forced desynchrony protocols (e.g., 24.18 h; ref. 16).

Measurements. Blood was sampled from an i.v. catheter 15 min into the rest section and 10 min into the exercise section of each exercise test. Blood was analyzed for epinephrine, norepinephrine, cortisol, whole blood platelet aggregability, and platelet count (the latter only in the last six exercise tests per subject), totaling 1,392 assays across all subjects (see *SI Methods* for details).

Blood pressure was measured by automatic oscillometric cuff sphygmomanometer (Dinamap; Critikon) every 5 min during each rest section and every 3 min during each exercise section, and averaged for the rest and exercise sections.

For assessment of HR and heart rate variability (HRV), three-lead ECG was recorded on a Vitaport (Temec Instruments) at 256 Hz throughout each exercise test. HRV analyses were performed according to the standards of the Task Force (48). For the analysis of HRV, cardiac interbeat intervals were obtained by using a QRS wave detector based on amplitude and first derivative of ECG waveform and verified by a trained technician to ensure that only “normal-to-normal” R waves times were included (49). Power spectra were calculated by fast Fourier transform of 3.41-Hz cubic spline resampled data across time windows with stable signal of at least 5 min for each rest and exercise section. High frequency power (HF; 0.15–0.4 Hz) and pNN50 (% of consecutive normal-to-normal R wave intervals differing >50 ms) were obtained as frequency- and time-domain vagal cardiac markers, respectively.

Statistical Analysis. Based on pre hoc criteria to eliminate spurious outliers, values > 3 SDs from the mean for each condition (rest or exercise) within each subject across all wake periods were excluded from analysis. CBT was measured continuously throughout the 240-h FD protocol by using a rectal thermistor (Yellow Springs Instrument) and assessed by nonorthogonal spectral analysis technique to determine circadian phase (timing) and period

(cycle length) as published (16). All data were assigned a circadian phase (0–359°) depending on the time from the fitted CBTmin (0°) and the individual's estimated circadian period (one full circadian period = 360°). Reactivity to exercise was calculated as the value of each variable during exercise minus the value during rest (reactivity = exercise – rest). The independent effects of the circadian cycle during rest (Hypothesis 1) and in the reactivity to exercise (Hypothesis 2) were assessed for each variable by using cosinor analyses including both the fundamental (≈ 24 -h) and second harmonic (≈ 12 -h) of circadian rhythmicity and two-factor mixed model analysis of variance with restricted maximum likelihood estimates of the variance components (JMP; SAS Institute) (50). Rather than assuming the data would fit a sinusoidal

waveform (i.e., the fundamental component), the inclusion of a ≈ 12 -h component (i.e., the second harmonic) permits fitting of a more complex function. For graphing purposes, to correct for any interindividual differences, data were expressed as a percentage of each individual's rest values across the FD protocol (left y axis in Fig. 2), in addition to the absolute values (right y axis in Fig. 2).

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Supporting Information

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SI Methods

Supporting Information on Exercise Test Battery. On the first baseline day, subjects performed an initial test exercise session on the ergometer cycling at 70 rpm to determine the personalized workload to achieve a target HR level of $\approx 60\%$ of the estimated maximum heart rate (HR) (using the formula: $220 - \text{age}$). Because we wanted to test the physiological response to an identical workload at different circadian phases and because the HR during exercise could be different at different circadian phases, subjects performed each subsequent exercise test with this same and constant workload at 70 rpm.

Supporting Information on Blood/Plasma Assays. Plasma epinephrine and norepinephrine were assayed by RIA (IBL America; sensitivity: 1 and 40 pg/mL, respectively) and cortisol by chemi-

luminescent assay (Beckman Coulter, Access; sensitivity: 0.4 $\mu\text{g}/\text{dL}$). For whole blood platelet aggregability, 450 μL of whole blood (samples collected in sodium citrate tubes) was diluted with 450 μL of physiological saline (NaCl) at 37 $^{\circ}\text{C}$. Platelet aggregability was assessed within 20 min of the blood draw as the area under the curve for the impedance signal ($\text{Ohm} \times \text{min}$) for platelet aggregability across the 10 min after stimulation with 5 $\mu\text{g}/\text{mL}$ collagen in duplicate by dual channel whole blood/optical lumi aggregometer (560VS; Chrono-Log) and analyzed with Chrono-Log software (Aggro/Link for Windows). For quality control, aggregometry data were excluded if duplicates differed by $>25\%$ from the mean (excluding 18% of data), resulting in a coefficient of variation of 7%. Platelet count was analyzed by FACSCalibur (Becton Dickinson) flow cytometer, as described (1).

1. Linden MD, et al. (2004) Application of flow cytometry to platelet disorders. *Semin Thromb Hemost* 30:501–511.

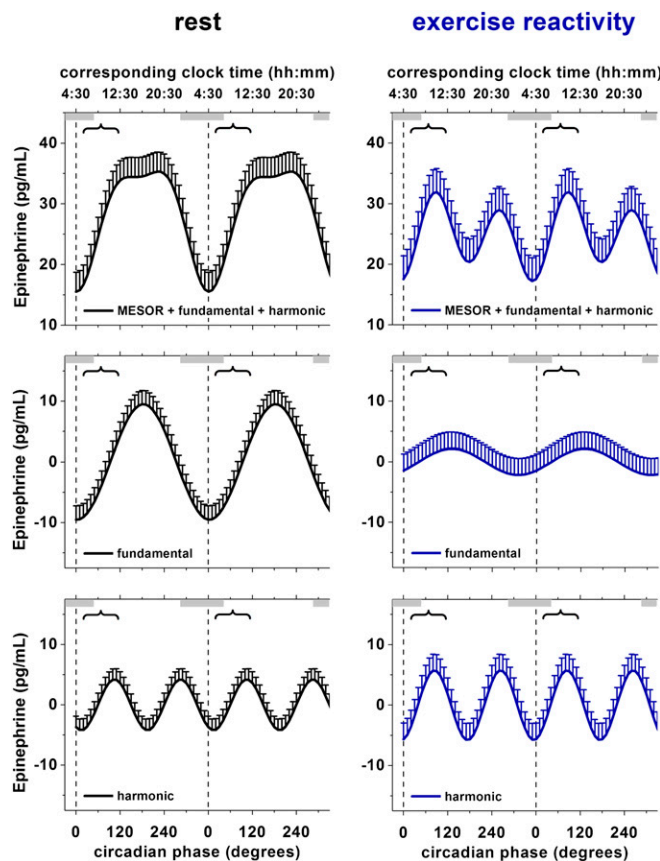


Fig. S1. Example of Cosinor model fit to circadian data. Shown are the cosinor fits to plasma epinephrine data at rest (Left) and cosinor fits to the change from rest induced by standardized exercise (exercise reactivity; Right). Each model reflects the summed MESOR, fundamental, and harmonic components (Top), the fundamental component alone (Middle), and the harmonic component alone (Bottom). Lines, model fit; error bars, SEM; gray bars, group average timing of habitual sleep episodes; vertical dotted lines, timing of the core body temperature minimum as circadian phase marker; curly brackets, most vulnerable period for adverse cardiovascular events observed in epidemiologic studies ($\approx 6:00$ AM–noon).

rest

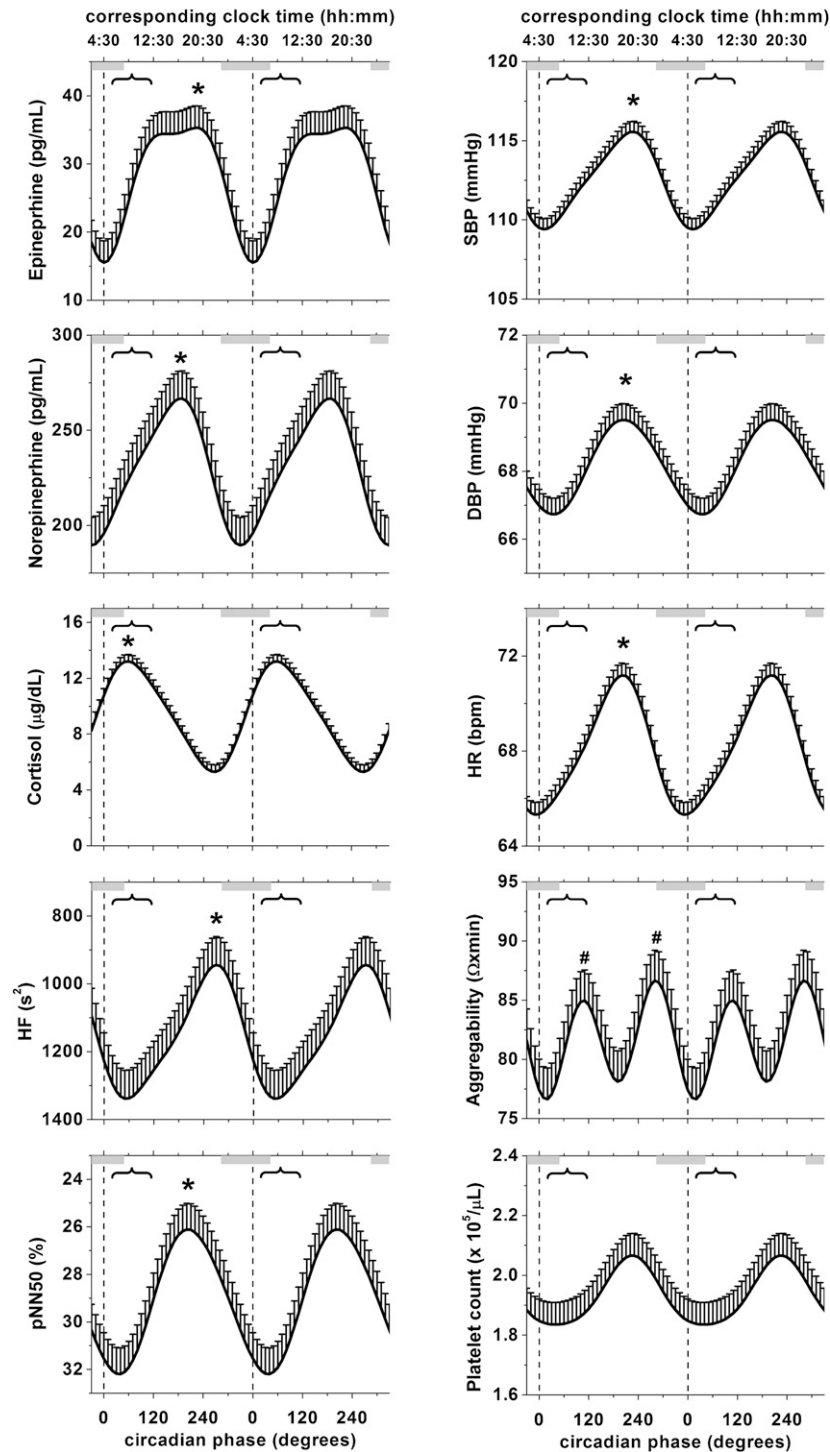


Fig. S2. Influence of the endogenous circadian system on cardiovascular function at rest. Cosine models of variables at rest revealed a significant fundamental circadian rhythm for epinephrine, norepinephrine, cortisol, HF, pNN50, SBP, DBP, and HR, and a harmonic rhythm (i.e., with a period of ≈ 12 h) for platelet aggregability. Platelet count did not show significant rhythmicity. Note, so that higher putative risk is upwards for all variables, the y axes for the vagal markers HF and pNN50 (*Bottom Left*) are inverted, i.e., upwards corresponds to vagal withdrawal. This is different from the orientation of these variables in Fig. 2 of the main manuscript. Symbols as in Fig. S1. *Timing of the peak for significant fundamental circadian cycle; #timing of the peaks for significant second harmonic of circadian cycle.

Exercise reactivity

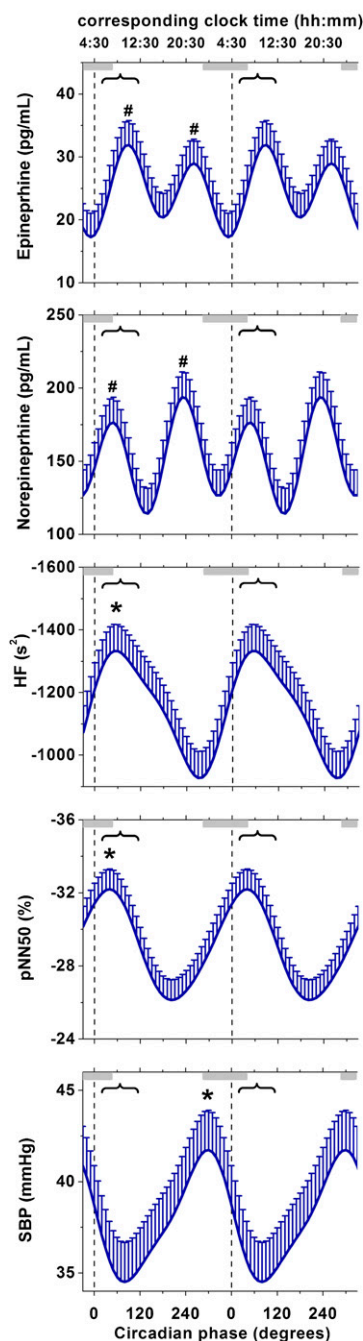


Fig. S3. Influence of the endogenous circadian system on cardiovascular reactivity to exercise. Cosine models of reactivity to exercise revealed significant circadian rhythmicity for HF, pNN50, and SBP, and significant second harmonics of the circadian rhythmicity for epinephrine and norepinephrine. The other variables did not have statistically significant rhythmicity ($P > 0.05$). Although a circadian variation in the magnitude of reactivity to exercise (vertical difference between red and black line) can be observed in Fig. 2, the statistically significant 12-h rhythmicity is more apparent in this figure because this is based on the cosine model that uses circadian phase data with a resolution of 1 circadian degree, whereas Fig. 2 shows the data grouped into 60° windows, which causes slight visual differences that do not affect the statistical analysis (all statistical analysis based on cosinor models). Note, so that higher putative risk is upwards for all variables, the y axes for the vagal markers HF and pNN50 are inverted, i.e., upwards corresponds to vagal withdrawal. This is different from the orientation of these variables in Fig. 2 of the main manuscript. Symbols as in Fig. 2. *Timing of the peak position for significant fundamental circadian cycle; #timing of the peak positions for significant second harmonic of circadian cycle.

