Circadian Differences in Stress-Induced Pressor Reactivity in Mice

Iveta Bernatova, Mary P. Key, James B. Lucot, Mariana Morris

Abstract—The objective of this study was to determine the effect of chronic stress exposure on the circadian pattern of cardiovascular responses in mice. Using male C57BL6 mice with carotid arterial catheters, we tested the effect of 7 days of intermittent shaker stress on body weight, food intake, drinking activity, plasma corticosterone, mean arterial pressure (MAP), and heart rate. The stress was delivered automatically for 2-minute periods (150 cycles/min), 45 times/d for 7 days. Plasma corticosterone was significantly increased in acutely and chronically stressed mice, with a partial attenuation in the chronic condition. Stress increased water intake, produced no change in food intake, and significantly decreased body weight (5% change). MAP and heart rate were measured continuously on stress days 1, 3, and 7 and during the basal and recovery periods. Chronic stress did not produce a sustained increase in MAP; however, there was an increase in MAP during the first stress day and a decrease during the recovery period. There was a circadian pattern in the pressor responses, with greater increases seen during the light period (nonactive phase) than in the dark period (+24% versus +11% on stress day 3, light versus dark). The results suggest that a stress delivered during the nonactive phase represents a higher cardiovascular risk. (Hypertension. 2002;40:768-773.)

Key Words: blood pressure  heart rate  corticosterone  circadian rhythm

Lifestyle stress is a risk factor for human diseases, including cancer, stroke, psychological disorders, and heart disease. There is a circadian pattern in the incidence of cardiovascular pathologies, with a higher frequency of heart attacks, strokes, and arrhythmias during the morning hours.1–3 Stressful conditions may also be a precipitating factor in the occurrence of cardiovascular accidents.

In animals, stress exposure produced by a variety of methods provokes a cascade of autonomic adjustments characterized by increased blood pressure (BP), increased heart rate (HR), and behavioral alterations. Most studies of stress biology have been conducted in rats, providing information on changes in cardiovascular function and eating and drinking behaviors.4–10 However, one complication of the experimental paradigms is that the stress effects can be enhanced by animal handling, noise, or pain, as seen in the forced swim test, air jet exposure, and physical restraint.4,8,11

Shaker stress is a mild, pain-free stimulus that elicits reproducible changes in BP, HR, sympathetic activity, and stress hormone secretion.12,13 There is no information on the application of shaker stress in mice and no data on the long-term effects of shaker stress on the cardiovascular system or on drinking and eating patterns.

For investigations in mice, we developed a computerized system for chronic, continuous BP recording and combined this with electronic recording of licking activity for analysis of circadian patterns.14,15 This methodology was applied to the investigation of the effects of stress on BP and HR responses and drinking behavior during the active (dark period) and nonactive (light period) phases in normotensive mice. For this purpose, we also set up a model for chronic stress exposure in mice, using shaker stress that was delivered remotely and automatically in the animal’s home cage.

Methods

Animals
Male C57BL6 mice (Harlan Inc, Indianapolis, Ind), 10 to 12 weeks of age, were used. All animal experiments were approved by Wright State University’s Laboratory Animal Care and Use Committee. The mice were housed at 22°C with a 12:12-hour dark-light cycle (5 AM to 5 PM, lights on). Animals were housed individually in clear cylindrical cages with a metal grid flooring, which covered standard bedding. Mice were maintained on a standard pellet diet (Harlan Teklad, 0.5% sodium by weight) and tap water ad libitum.

Shaker Stress
For shaker stress, a specially designed caging system was attached to a shaking platform (Model 5901; Eberbach Inc). The shaking device was programmed to provide intermittent shaker stress, 2 minutes of shaking (150 cycles/min; 2.86 cm stroke) followed by variable rest periods from 13 to 45 minutes. The variable rest periods between stress sessions were chosen with the aim of introducing an unpredictable stress stimulus. The same paradigm of 45 shaking sessions/24 h was used during all 7 days of stress.
Plasma Corticosterone

Corticosterone levels were compared in mice exposed to acute or chronic stress. Seventy-four mice were divided into 3 groups. In the acute group, 28 mice were exposed to a single 2-minute shaking session. In the chronic group, 30 mice were exposed to chronic shaker stress for 7 days. Sixteen mice served as controls, with the same handling but without stress. Mice were killed 5, 30, or 60 minutes after the last shaking session (n=9 to 10/group) between 9 and 11 AM. After decapitation, trunk blood was collected in heparinized test tubes and centrifuged; plasma samples were stored at −30°C. Plasma corticosterone was determined with the ImmunChem double antibody corticosterone 125I RIA kit (ICN Biochemicals Inc).

Body Weight, Food Intake, and Drinking Activity

Basal data were recorded for 4 days, followed by 7 days of intermittent shaker stress and 3 days of recovery. Body weight (BW), food intake (n=10), and water intake (n=5) were measured daily. Drinking activity was recorded with a drinkometer system15 (Columbus Instruments) interfaced with a computerized data acquisition system (Biopac Inc). Incidental contacts of mice with the water bottles, registered as single data points, were excluded from the analysis. Food intake was measured by daily weighing of the food.

Cardiovascular Measurements

Mice were prepared with chronic carotid arterial catheters according to the method of Li et al. After surgery, a heparinized saline solution (100 U/mL) was continuously infused into the catheter at 25 μL/h with the use of a syringe pump (model 220, KD Scientific). The pressure produced by the infusion pump modified BP readings by approximately 2.9 mm Hg. The catheter was covered with a metal spring that was attached to a fluid swivel at the top of the cage. The animals were allowed to recover from surgery for at least 5 to 6 days, by which time water and food intake had returned to normal. BP and HR were recorded continuously (24 hours) on stress days 1, 3, and 7 and on the day before and after stress (basal and recovery).

Stress responses of mean arterial pressure (MAP) and HR to individual shaking sessions were evaluated on stress days 1, 3, and 7 and on day 1 of the recovery period during the light and dark periods (11 AM and 11 PM). Responses were calculated as a percentage of the values during the 2 minutes preceding the stress.

Statistical Analysis

All results are presented as mean±SEM. One-way ANOVA and least significant differences tests were used for analysis of BW and food intake. Two-way ANOVA and Newman-Keuls tests were used for analysis of the stress effect on corticosterone levels and drinking activity. Differences in MAP and HR in the chronic experiment were evaluated by 2-way ANOVA followed by Duncan’s test. Stress responses were evaluated by 3-way ANOVA followed by the Newman-Keuls test. Values were considered to differ significantly if the probability value was <0.05. Circadian data of drinking activity, systolic BP, diastolic BP, and HR were recorded with a sampling rate of 85 samples/s and converted from digital to numeric form with the use of acquisition software. Data were processed by calculation of 10-minute means of the respective variable. These 10-minute means were averaged for calculation of the dark and light period means. Drinking data were then converted to text files, processed by Circadia software (Behavioral Cybernetics), and smoothed using 60-minute moving averages. Finally, data were converted to a circadian autogram and to the graph (MATLAB Graphics 5.2.1).

Stress responses of MAP and HR were recorded and analyzed in the same manner as circadian data of MAP and HR; however, data were processed by calculation of 2-minute averages that were used for calculation of MAP and HR values before, during, and after stress (divided into periods 1 to 8 minutes and 9 to 16 minutes after stress).

Results

Plasma Corticosterone

Corticosterone levels in control mice were 12±2.7 ng/mL. Stress produced significant increases in plasma corticosterone under acute and chronic conditions (F[2,65]=65.8, P<0.0001, Figure 1). The acute response was 2.5-fold greater than the chronic response, with peak levels of 132 and 53 ng/mL, respectively (P<0.0001). There were no time-related differences in plasma corticosterone in the acute experiment between 5, 30, and 60 minutes after stress. In chronically stressed mice, a 4-fold increase of corticosterone was found 30 and 60 minutes after stress compared with the control levels (P<0.01).

Body Weight and Food Intake

BW differed during basal, stress, and recovery periods (F[2,107]=3.21, P<0.05, main effect of stress, Table). Post hoc analysis showed a marginally significant decrease of BW on the third day of stress versus the last day of basal recording (P=0.06, decrease of 6%). There were no observed differences in food intake (Table).

Drinking Activity

Drinking activity was measured by continuous recording of licking activity during the basal, stress, and recovery periods. On

<table>
<thead>
<tr>
<th>Day</th>
<th>Body Weight, g* (n=10)</th>
<th>Food Intake, g (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal 1</td>
<td>27.22±0.54</td>
<td>4.42±0.18</td>
</tr>
<tr>
<td>Basal 2</td>
<td>27.34±0.59</td>
<td>4.86±0.15</td>
</tr>
<tr>
<td>Basal 3</td>
<td>27.59±0.68</td>
<td>4.76±0.18</td>
</tr>
<tr>
<td>Basal 4</td>
<td>27.78±0.67</td>
<td>4.17±0.30</td>
</tr>
<tr>
<td>Stress 1</td>
<td>26.43±0.58</td>
<td>4.65±0.17</td>
</tr>
<tr>
<td>Stress 2</td>
<td>26.33±0.59</td>
<td>5.23±0.32</td>
</tr>
<tr>
<td>Stress 3</td>
<td>26.13±0.58</td>
<td>4.76±0.11</td>
</tr>
<tr>
<td>Stress 4</td>
<td>26.26±0.59</td>
<td>4.82±0.11</td>
</tr>
<tr>
<td>Stress 5</td>
<td>26.41±0.56</td>
<td>4.76±0.11</td>
</tr>
<tr>
<td>Stress 6</td>
<td>26.54±0.61</td>
<td>4.77±0.11</td>
</tr>
<tr>
<td>Stress 7</td>
<td>26.59±0.63</td>
<td>4.66±0.13</td>
</tr>
<tr>
<td>Recovery 1</td>
<td>26.86±0.70</td>
<td>4.85±0.22</td>
</tr>
<tr>
<td>Recovery 2</td>
<td>27.21±0.80</td>
<td>4.87±0.22</td>
</tr>
<tr>
<td>Recovery 3</td>
<td>27.41±0.74</td>
<td>4.50±0.21</td>
</tr>
</tbody>
</table>

*Stress decreased body weight significantly (F[2,107]=3.21, P<0.04 for main effect of stress) compared to basal values. Values are mean±SEM.
the basis of these data, average waveforms were constructed that showed the diurnal pattern in drinking activity (Figure 2). Stress did not alter the diurnal pattern of drinking (Figures 2 and 3), which was concentrated in the dark period (F[1,128]=345.3, P<0.0001, main effect of circadian factor). Stress increased significantly drinking activity of mice in the dark period (F[1,128]=5.90, P<0.02, interaction stress x circadian factor) as compared with nonstress periods (basal and recovery).

**Blood Pressure and Heart Rate**

MAP and HR were analyzed with the use of a program that compiles all of the data over the 12-hour dark/light periods (3.7×10⁶ samples/12-hour period). Basal MAP in the stress group was 111±0.98 and 104±1.15 mm Hg during dark and light periods (12-hour averages), respectively (Figure 4). There was a significant light/dark rhythm in MAP (F[1,54]=63.2 P<0.0001, main effect of circadian factor), with highest levels noted during the dark period. Analysis of the time-related changes showed significant alterations (F[4,54]=17.9, P<0.0001, main effect of experiment day), with an increase in MAP (24-hour average) on the first day of stress (P<0.008) and a decrease during recovery (P<0.001 versus basal value). A significant increase in MAP occurred in the light period of stress day 1 (versus basal/light), whereas there were decreases in both light and dark periods of recovery (versus the appropriate basal period, Figure 4).

The basal HR of mice was 600±13 and 565±10 beats/min during dark and light periods (12-hour averages), respectively (Figure 4). Analysis of the time-related changes showed a decrease in HR (24-hour average) on the last day of stress (P<0.003) and during recovery (P<0.007) versus the basal day. Circadian analysis revealed that the significant drop of HR occurred in the dark period of day 7 of stress and recovery versus basal/dark (Figure 4).

**Stress-Induced Cardiovascular Responses**

To evaluate cardiovascular reactivity, we examined the pressor and heart rate responses during the light/dark periods on days 1, 3, and 7 of chronic stress. The results showed that there was a diurnal pattern in stress-induced pressor reactivity. Stress delivered during the light period increased MAP significantly more than stress delivered in the dark period (F[1,162]=30.0, P<0.0001, main effect of circadian factor; Figure 5). The immediate increases of MAP during the stress event in the light period were ≈26%, 24%, and 23% (P<0.001) on days 1, 3, and 7, respectively, as compared with basal. The immediate increases of MAP during the stress event in the dark period were 16% (P<0.001), 11% (P=0.16), and 21% (P<0.01) on days 1, 3, and 7, respectively. In both light and dark periods, MAP gradually decreased after stress (F[2,162]=23.3, P<0.0001, main effect of the time course, ie, stress period, 1 to 8 minutes and 9 to 16 minutes after stress).

**Figure 2.** Circadian pattern of drinking activity, comparing basal (average waveform for 4 days of basal recording), the first day of stress, and the third day of the recovery period. Heavier part of x-axis represents dark period. Values represent average waveform (dark line) and average waveform positive error (light line).

**Figure 3.** Effect of chronic intermittent shaker stress on drinking activity. B1 through B4, Days of basal recording; S1 through S7, days of stress; R1 through R3, days of poststress recovery. Drinking was significantly concentrated in the dark period (F[1,128]=345.27, P<0.0001). Stress significantly increased drinking activity of mice in the dark period (F[1,128]=5.90, P<0.02 for interaction of stress and circadian factor) compared with nonstress periods (basal and recovery) without alterations during the light period. Values are mean±SEM.

**Figure 4.** MAP and HR of stress-exposed mice. Bas indicates basal day; S1, S3, and S7, days of stress; Rec, day 1 of poststress recovery. P<0.01 vs dark period on the same day; *P<0.05 vs basal/light; †P<0.05 vs basal/dark. Values are mean±SEM.
There were no differences in HR responses during the dark/light periods (Figure 6). The immediate increases of HR during the stress event were not different as compared with basal values. Significant differences between the dark and light period response were observed on day 3 ($P < 0.05$).

**Discussion**

The objective of the present study was to characterize a model of chronic shaker stress exposure in mice and to investigate the effect of shaker stress on the circadian pattern of cardiovascular responses. The key findings are (1) chronic shaker stress provides a viable model for the investigation of stress effects in mice; (2) stress produced alterations in MAP, with an increase noted on the first day of stress; and (3) there was a diurnal pattern in pressor reactivity with prominent differences noted between daytime and nighttime exposures.

Long-term shaker stress increased plasma corticosterone, although the activation of the hypothalamic-adrenal axis was partially attenuated as compared with the acute stimulus. There was a $>$4-fold increase in plasma corticosterone after 7 days of stress (compared with controls), which suggests that shaker stress remained an effective stimulus suitable for long-term studies. Adaptation to stress is a normal response, since without physiological compensation there could be escalating, detrimental effects on the organism. Indeed, for repeated restraint stress, social stress, noise stress, and forced swimming, there is a habituation of corticosterone responses. However, in contrast to our results, a study in rats showed no attenuation of the corticosterone response to repeated shaker stress. The difference in the results may be related to the level of stimulation and/or the animal species. Hashiguchi et al. tested the effect of daily shaking sessions delivered over 14 days, whereas in our experiment, mice were exposed to 315 shaking sessions over a 7-day period. Taking into consideration the number of stress sessions administered and the time course of the plasma corticosterone response, it is likely that there is a generalized increase in adrenal steroid secretion in the chronically stressed mice.

Various models have been used to study stress/cardiovascular interactions in experimental animals. In the majority of these experiments, subjects were exposed daily to a single stress event and the cardiovascular responses were determined over limited time periods after the stress session. However, chronic paradigms are more relevant to the human situation in which people are exposed to multiple small stressors over long periods of time. Another issue is that most of the experimental stress models include the possibility of exposure of the animals to other stressful stimuli, such as handling, noise, and vibration. We used shaker stress as the test paradigm because it elicits reproducible cardiovascular changes, and with our specialized caging system it is easy to administer to the conscious animal without...
additional handling. In previous studies, 5-minute of acute shaker stress (150 cycles/min) in rats produced significant increases in MAP and HR that persisted 5 minutes after the end of the shaking.12

There is less information on the effects of chronic stress on cardiovascular parameters and no information on the effects of chronic shaker stress. In this study, MAP and HR were recorded continuously for 24-hour periods on selected days of the stress exposure. Intensive monitoring is important because it provides an accurate picture of acute cardiovascular responsiveness as well as long-term changes. The composite data show that there were no sustained increases in BP during chronic shaker stress. However, an increase of MAP was noted on the first day of stress and a decrease on the first day of recovery. It is interesting that the changes occurred during the initiation and termination of the stress, suggesting that a rapid adjustment in autonomic/endocrine outflow occurred at these times.

Several studies have addressed the problem of stress-induced hypertension. However, there are conflicting data as to the nature of the cardiovascular changes induced by these stressors. A social stress paradigm in which male rats were housed with different females produced no change in basal blood pressure or its circadian rhythm.21 A chronic multiple stress paradigm produced increases in HR, HR variability, and stress-induced tachycardia but no changes in BP.22 Other studies demonstrated interactions between stress and genetic background. Henry and colleagues23 presented evidence that chronic social stress, produced by group housing, caused an increase in blood pressure in Long-Evans rats but not in Wistar-Kyoto rats. Similarly, in spontaneously hypertensive rats, restraint-induced tachycardia and pressor responses were greater than in the normotensive Wistar rats.8 Lawler et al24 reported that borderline-hypertensive rats showed a greater pressure response to conflict stress as compared with normotensive strains. Thus, genetic factors may play a crucial role in cardiovascular regulation and the development of stress-induced hypertension.

In mice, psychosocial stimuli produced prolonged hypertension.25 However, this study used a tail-cuff method for determination of blood pressure, which exposes the mice to additional "acute stressors" associated with the recording method (handling, noise, heat, restraint, and pain). In this situation, the pressor response of chronically stressed mice to "acute stress" during BP recording may be greater than in controls as the result of sensitization of cardiovascular responses.26 Thus, an appropriate method of BP recording is necessary for accurate evaluation of cardiovascular responses to stress exposure. The chronic arterial catheter and data acquisition system used in the present study allows for recording BP and HR in undisturbed mice during stress sessions and rest periods. The catheter system also permits painless blood sampling for the evaluation of stress hormone levels (not available using radiotelemetry). This prevents any hyperresponsiveness caused by handling and novelty.

The analysis of the circadian pattern of stress responsiveness revealed some interesting findings. There was a marked increase in the pressor response when the stress was delivered during the light phase (nonactive, sleeping period). In fact, on the third day of stress, there were no significant changes in BP during the dark (active) period but a 24% increase during the light period. An examination of the behavioral activity associated with chronic stress showed a similar circadian pattern.27 Stress significantly decreased locomotor activity but only during the day. The reason for the difference in the day/night cardiovascular and behavioral responses is not known but may be related to sensory activation (waking from sleep), changes in regional hemodynamics, input from circadian pacemakers, or other factors. Certainly, in humans there is considerable evidence linking the circadian period to cardiovascular events such as myocardial infarctions and strokes. For example, there was a peak in cerebrovascular strokes in humans in the morning (6 AM to noon) and a decreased incidence during the night (6 PM to 6 AM).13 The morning onset of cardiovascular events may be associated with increased sympathetic activation as well as the stress from daily activities. These epidemiological studies were, however, focused on the resultant pathology rather than cardiovascular responsiveness, as investigated in our animal study.

The time course of BP alterations observed during shaker stress suggested sudden increases of BP during stress events, with a decline to basal or even below basal values during rest periods. Even though the pressor responses are of relatively short duration, these repeated fluctuations could have pathological consequences. On the one hand, changes in arterial pressure lead to corresponding alterations in shear stress that can increase nitric oxide (NO) release from the endothelium and at least partially buffer the pressure overload.28 On the other hand, sudden pressure fluctuations could damage the endothelial monolayer and result in reduced endothelium-dependent NO release. Thus, a functional endothelium may be especially important during chronic stress. Indeed, chronic social conflict increased the rate of endothelial cell damage and reduced NO bioavailability in the coronary arteries of primates.29,30 In humans, brief episodes of mental stress induced transient endothelial dysfunction in healthy young adults,31 which may represent a link between stress and atherogenesis.

For evaluation of stress-induced metabolic changes, we studied water and food intake and BW. Despite the increase in water intake and the lack of change in food intake, chronic stress caused a small but significant loss of BW. This decrease in BW is presumably associated with increased metabolic demands, reduced digestion, and increased adrenal steroid secretion.32 There are various reports of a stress-induced alterations of BW: decrease in BW,33,34 no change in BW,30 and even an increase in BW.35 Similarly, variable effects of stress on food and water intake were observed.36,37 In terms of the circadian pattern of water intake, the increase in drinking (recorded as increased licking activity) produced by chronic stress occurred primarily during the dark period. However, the stress did not change the diurnal rhythm of water intake, even though there were experimentally imposed disruptions in the sleep cycle. Thus, alterations of BW and food and water intake in the different experimental studies probably are dependent on the type of the stressor, its duration, and animal species and sex.

In conclusion, we developed and characterized a model for chronic stress exposure in mice that allows for the determination of BP and HR in the undisturbed state. The results showed that stress produced an activation of the hypothalamic-adrenal axis, which was partially attenuated in the chronic condition. There was no sustained increase of MAP, but there were important circadian changes in pressor responsiveness. The study provides evidence that stress...
delivered during the nonactive phase of the day represents a higher cardiovascular risk than stress delivered during the active phase. This may have implications for the human condition, in which there are circadian patterns in the incidence of cardiovascular problems as well as links between stress and heart disease.

Perspectives
Stress, defined as a physical, chemical, or emotional factor that causes bodily or mental tension, is a risk factor in a variety of diseases. Because stressful conditions are often chronic, it is important to have animal models for examining the pathological processes. In this paper, we report on the characterization of a model for chronic stress exposure in mice. Intermittent shaker stress provides a unique pain-free model of emotional and physical stress. It can be administered remotely and is easily combined with data collection systems such as those for cardiovascular, ingestive, and endocrine function, allowing for a comprehensive evaluation of its effects. Studies in humans suggest that stress augments and/or initiates cardiovascular pathologies. There is also evidence for day/night rhythms in the incidence of myocardial infarctions and stroke, with greater occurrences during the early waking hours. We report that in mice, there are circadian variations in stress-induced cardiovascular responses. This is seen as increased pressor responses during the day period when the animals are sleeping. This data may be relevant to the human condition because it suggests that stress presented during the inactive period represents a greater cardiovascular risk.

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