Enhanced heart rate variability and baroreflex index after stress and cholinesterase inhibition in mice

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PYRIDOSTIGMINE (PB), a reversible inhibitor of acetylcholinesterase (AChE), is used clinically for the treatment of autoimmune disease and prophylactically against organophosphate poisoning (9, 32, 36). Generally, it causes few overt symptoms and is thought to be a safe drug (8, 19, 40). However, there are questions as to whether PB may have central interactions under stressful conditions or may have access to brain areas lacking a sufficient barrier. Forced swimming or immobilization disrupted the blood-brain barrier (BBB) and allowed access of PB into brain tissue (17, 48). Chronic PB exposure in mice led to a decrease in AChE activity in the hypothalamus, a brain region with a reduced BBB (45). PB also modified the response to novelty stress in rats and mental stress in humans (28, 40, 47). These observations suggest that the combination of operational/environmental stress and PB exposure might cause unexpected health effects, raising questions about a possible role of PB in the etiology of the symptoms collectively termed Gulf War illness (25).

Reduced heart rate (HR) variability (HRV) is associated with a variety of cardiovascular pathologies, from myocardial infarction to heart failure (18, 34). The overall magnitude of HRV can be easily quantified by measurement of standard deviation of the beat-by-beat series of pulse interval (PI). However, spectral analysis provides more detailed and specific information on the frequency-domain characteristics of PI variability, including data on sympathetic and parasympathetic modulation of cardiac function (38). There is evidence from studies in patients with congestive heart failure that reduced low-frequency power (LF) was an independent predictor of sudden cardiac death (18, 34). Even spectral analysis of short-term electrocardiogram measurements were useful in identifying patients at greatest risk who might benefit from further treatment (34).

Stress, defined as any physical or emotional influence that causes bodily or mental tension, results in a cascade of cardiovascular, endocrine, and immune changes. Clinical studies suggest that mental stress is associated with adverse cardiovascular events such as myocardial ischemia, arrhythmias, infarction, and stroke (14, 20, 43, 44, 53). In an extensive clinical study, cardiac patients were exposed to exercise or psychological stress (49). Results showed that mental stress-induced ischemia was a predictor of sudden death. With the use of an actual lifestyle stress, a medical school examination, Lucini et al. (37) found that stress was associated with impairment in cardiac and enhancement in vascular responses. The results of this study verified the importance of measurement of a spectral profile rather than simple time-domain variance.

To extend the results of clinical studies, animal models have been developed to investigate the pathophysiological effects of stress. In many cases, the stressors are physical insults, such as restraint, exhaustive swimming, or electric footshock. We used a chronic stress model that combines shaking (a physical stimulus) with novelty (intermittent exposure) (7). It produces consistent, repeated increases in blood pressure (BP), HR, and corticosterone secretion (7, 33).
Experiments were designed to explore the interactions between psychosocial stress exposure and cholinesterase (ChE) inhibition on the 24-h pattern of BP and HR and its respective spectral components in mice. The idea was to emulate some of the conditions of the Gulf War in which soldiers self-medicated with PB tablets while being exposed to environmental stressors. The hypothesis is that the dynamic sensitive indexes of HRV and pressure variability might be predictive of the problems associated with this syndrome.

MATERIALS AND METHODS

Animals. Male C57BL/6J mice (Harlan, Indianapolis, IN), 26–27 g, were housed individually at 22 °C on a 12:12-h light-dark cycle. They were given a standard diet (Harlan Teklad, 0.4% sodium by weight) with tap water ad libitum. The Laboratory Animal Care and Use Committee of Wright State University approved all experimental protocols.

Surgery. Under ketamine-xylazine anesthesia (120:20 mg/kg im), mice were prepared with chronic carotid arterial catheters according to methods previously described (7, 35). Heparinized saline (100 IU/ml) was continuously infused intra-arterially (25 μl/h) to maintain catheter patency. Animals were allowed at least 6 days to recover from the cardiovascular surgery before experimentation. Osometric minipumps, filled with saline or PB (Alzet model 1007D, flow rate 0.5 μl/h, DURECT; Cupertino, CA), were inserted subcutaneously (ketamine-xylazine anesthesia) after completion of the basal cardiovascular recordings (Basal).

Cardiovascular recordings. All recordings were made in conscious mice while in their home cages. The catheter was connected to a flow-through pressure transducer (model 041–500503A, Argon; Athens, TX), which was connected to a computerized data-acquisition system (model MP100WSW, BIOPAC Systems; Santa Barbara, CA). Arterial BP (AP) was sampled at different rates for 24-h recording and spectral analysis (80 and 4,000 Hz, respectively). HR was derived from AP data.

In the groups exposed to shaker stress (Stress and PB/Stress), cardiovascular recordings were made under basal conditions (Basal) and after 3 days of stress (day 3). The latter recording was begun 30–40 min after the last stress exposure. This protocol was based on the time course of cardiovascular changes produced by 2 min of shaker stress. There was an immediate increase in BP and HR, which returned to baseline 20–30 min later (data not shown). For 24-h recordings, BP and HR were continuously recorded before minipump implantation (Basal) and on day 3 of drug treatment and/or stress. The data were processed by calculation of 10-min means that were averaged for calculation of the dark-light levels. For spectral analysis, the pressure recordings were made for 20 min (0900–1100 hours) under Basal conditions and on day 3 of drug treatment and/or stress as described above.

The experimental groups (n = 5–6 mice/group) were as follows: 1) stress with saline infusion, 2) stress with PB infusion, and 3) PB (10 mg/kg –1day–1) infusion without stress. The stress paradigm (7) used intermittent shaker stress delivered in the home cage for 3 days (2 min stress, 150 cycles/min, 45 times/day). The interstress rest periods were variable (13–45 min) with the goal of adding unpredictable timing to the stress.

Spectral analysis. A Windaq Waveform Browser (Dataq Instruments; Akron, OH) was used to process AP data to extract beat-by-beat time series of PI and mean AP (MAP). The overall variabilities of the PI and MAP series in time domain were calculated and expressed as the standard deviation (SD) of the entire time series. PI and MAP fluctuations were assessed in the frequency domain using autoregressive spectral analysis (software provided by A. Porta, Milan, Italy). The theoretical and analytic basis for autoregressive modeling of oscillatory components has been described previously (3, 38).

Briefly, the PI and MAP series were divided in segments of 300 beats, overlapped by 50%. The spectra of each segment were calculated via the Levinson-Durbin recursion, and the order of the model was chosen according to Akaike’s criterion, with the oscillatory components quantified in the LF (0.1–1.0 Hz) and high-frequency (HF: 1.0–5.0 Hz) ranges (29). Spontaneous baroreflex sensitivity (BRS) was calculated using the α-index within the LF range (α-index = square root of the LF/HF-LF/2HF power ratio). The calculation of the α-index requires the presence of significant coherence between PI and MAP time series at the LF range. Therefore, to evaluate the coherence, a bivariate autoregressive analysis was performed between PI and MAP time series (42, 46). In the LF range, the coherence between PI and MAP is an expression of the baroreflex control of HR (38, 42, 46). The α-index was calculated in all cases because the coherence value was significant (k2 > 0.5).

Blood ChE. Total blood ChE, AChE, and butyrylcholinesterase (BChE) activities were determined before treatment (Basal) and on day 3 of treatment. ChE activity was determined by a modified colorimetric method (6, 13). Measurements were made in whole blood, which was collected from the arterial catheter. Blood samples were stored at 4 °C, and enzyme activities were determined within 4 h of collection. AChE activity was determined by inhibiting BChE activity with 25 μM tetraisopropylypyrophosphoramide (Sigma Chemical; St. Louis, MO). BChE activity was calculated by subtracting AChE activity from total ChE activity.

Statistical analysis. Results are expressed as means ± SE. ChE activities were compared using one-way ANOVA. Multi-way ANOVA was used to determine differences in day-night variations of MAP and HR (day cycle, group, and time as sources of variation). Two-way ANOVA for repeated measures was performed on all MAP/HR baseline values as well as their respective parameters of variability and baroreflex index data. There was no violation of circularity assumptions, as required for the use of two-way ANOVA for repeated measures. Tukey test was used for multiple comparisons. P < 0.05 was considered statistically significant.

RESULTS

MAP and HR were measured over 24-h periods with calculation of the day-night averages (Fig. 1). There was a significant effect of diurnal circadian cycle with increases noted during the dark/active period [F(1,60) = 6.97 and P = 0.01 for HR; F(1,60) = 16.82 and P = 0.001 for MAP]. There was no overall effect of stress or PB on the circadian patterns of MAP and HR; there were, however, individual stress responses over the 24-h period as shown in a previous publication (7).

For evaluation of PI variability, pressure was sampled over a shorter period of time (~20 min). There were significant treatment [F(3,81) = 6.56 and P = 0.024] and interaction [F(3,81) = 5.90 and P = 0.015] effects for PI variability in the time domain (SD). There were no differences between stress (5.0 ± 0.9 ms) and PB (7.5 ± 0.9 ms), but the combined treatment produced up to a twofold increase in SD (11 ± 1.4 ms, PB/Stress).

Figure 2 shows a representative PI time series and respective spectrum from each group in Basal and day 3 of treatment and/or stress (Stress, PB, and PB/Stress). Spectral analysis showed two distinct oscillations of PI, one in the LF and another in the HF band. The central frequency of the LF oscillation was 0.39 ± 0.06, 0.36 ± 0.06, and 0.32 ± 0.05 Hz in Basal and 0.44 ± 0.04, 0.30 ± 0.02, and 0.26 ± 0.02 Hz in day 3 recordings for the Stress, PB, and PB/Stress groups, respectively. The central frequency of the HF oscillation was 3.4 ± 0.2, 3.0 ± 0.3, and 3.5 ± 0.1 Hz in Basal and 3.6 ± 0.2, 3.6 ± 0.3, and 3.3 ± 0.2 Hz in day 3 recordings for the Stress,
PB, and PB/Stress groups, respectively. The frequencies of the oscillations were similar among groups in Basal as well as day 3 recordings. Shaker stress alone or PB treatment did not alter the frequency or power of either LF or HF oscillatory components. On the other hand, spectral analysis showed a marked increase in the power, but not frequency, of the LF oscillations of PI when PB treatment was coupled with chronic stress (Figs. 2 and 3). The LF peak in the example reached a level of >4,000 ms²/Hz (power), which required a graphic inset with an expanded y-axis. When all group values of LF power were compared (Fig. 3), there were significant effects of treatment $[F(3,81) = 4.87$ and $P = 0.046]$ and interaction $[F(3,81) = 4.93$ and $P = 0.025]$, and a difference was found only for the PB/Stress group (9.5 ± 1.7 compared with 3.3 ± 0.9 and 5.0 ± 0.6 ms in the Stress and PB groups, respectively).

The spectral analysis of MAP revealed two distinct LF and HF oscillatory components at frequencies similar to the PI oscillations (Table 1). There were no differences in the power of MAP oscillations in the groups (Table 1). Bivariate spectral analysis between PI and MAP revealed high coherence values (0.67–0.96) in both LF and HF range. The $\alpha$-index calculated in the LF range was not affected by stress (1.8 ± 0.3 ms/mmHg) or PB treatment (1.7 ± 0.5 ms/mmHg), but it increased tremendously when PB treatment was combined with chronic shaker stress (3.6 ± 0.5 ms/mmHg; Fig. 4).

Blood ChE and AChE activities were reduced in the mice treated with PB (Fig. 5), but there was no effect of stress. BChE was not altered by the treatment.

**DISCUSSION**

The idea that psychosocial stress can modify the effects of pharmacological agents, resulting in enhanced or reduced effects, has received much attention. In retrospective analysis of the “Gulf War syndrome,” there was speculation that stress interacts with the ChE inhibitor PB to accentuate the pathology (23–25). While there is experimental information on the behavioral, enzymatic, and receptor alterations associated with stress and PB treatment, there are less data on the cardiovascular axis (1, 4, 17, 51). We approached the problem by testing the effect of stress and PB exposure on the 24-h pattern of BP and HR and the associated variability in the time and frequency domains. Results were surprising in that there were no changes in absolute BP or HR levels in any of the groups, but there were dramatic increases in variability when PB treatment was coupled with stress exposure. This was seen as an increase in the HR spontaneous variations in both time and frequency domains and an increase in baroreflex index. The changes in the HRV were specific for the LF range, which is associated with autonomic and baroreflex function in mice. These results demonstrate 1) the utility of a detailed statistical analysis of cardiovascular oscillatory patterns for functional evaluation and 2) evidence for potent interactions between environmental stressors and cholinergic systems, raising questions about the global use of PB.

There was increased interest in PB and its physiological effects after the conclusion of the 1992 Persian Gulf War (9, 23–25, 32). This was a situation in which large numbers of healthy, young people were given PB chronically. The military personnel were required to take PB (45 mg po 3 times daily) as a prophylactic against chemical warfare agents. With the appearance of the so-called Gulf War syndrome in the years after the military engagement, there was speculation that PB had toxic side effects that were not observed under normal conditions (23, 24). Experimental studies explored the idea that stress, a normal condition of military deployment, might alter the effects of PB. Friedman et al. (17) showed that swim stress increased the entry of PB into brain tissue and activated neuronal systems. Later, they verified that the treatment produced long-lasting changes in cholinergic neuronal expression (31). However, these findings were not universally accepted because others failed to find significant interactions between PB and stress as measured by behavior and ChE activity (21, 30, 51). One investigation even reported an enhancement, rather than a reduction, in AChE activity after PB/stress (50). We studied the effect of chronic PB infusion in mice and found that there were regional changes in brain ChE activity (45). AChE was reduced or increased in the hypothalamus but unchanged in the cerebral cortex. There were no changes in behavioral parameters as related to locomotion and no changes in BP or HR (6). Beck et al. (4) reported a small change in cortical AChE activity in animals subjected to a PB/electric shock combination. PB may also interact with the central nervous system (CNS) via effects on afferent nerve traffic as demonstrated by a study that showed that PB produced rapid changes in stress-induced hyperthermia (28).

Investigations in humans have focused on the idea that PB may have beneficial effects, related to HRV, and might be used as a treatment for heart disease. There is evidence that autonomic dysfunction, associated with increased sympathetic...
Fig. 2. A: time series of pulse interval (PI; in ms) for one representative mouse of each experimental group during basal conditions and 3 days after stress, PB, or PB/stress. The respective spectra are shown in B. The standard deviation (SD; in ms) of PI variability is shown above the time series (means ± SE). The graphic inset with an expanded y-axis was necessary for the PI spectra of the PB/stress mouse on day 3 because of the large low-frequency (LF) power. *P < 0.05 vs. basal in the PB/stress group.

Fig. 3. Power spectral density (in ms) of LF (0.1–1.0 Hz) and high-frequency (HF; 1–5 Hz) components of PI variability calculated by spectral analysis for each experimental group [stress (A), PB (B), and PB/stress (C)] during basal and after treatment. *P < 0.05 compared with basal.
drive and reduced vagal modulation, may play a role in heart failure. Indeed, reduced HRV is a predictor of increased mortality risk in patients with heart disease (18, 34). Behling et al. (5) tested the effect of PB treatment in patients with heart failure. They reported a 65% reduction in ventricular ectopic activity and an increase in HRV and suggested that long-term clinical trials were warranted. Studies in healthy subjects showed that PB treatment increased HRV (41) and interacted with mental stress to produce increased left ventricular outflow velocity (47). Thus results indicate that PB may act in the periphery, on nerve endings, to stimulate acetylcholine release and modulate cardiac function.

Spectral analysis techniques have been applied to the study of cardiovascular function in humans and rodents. The basic premise is that BP variability and HRV studied in the frequency domain provides information on autonomic modulation of cardiovascular system and may be used to calculate spontaneous baroreflex activity (2, 3, 12, 26). In humans, the LF-to-HF ratio is used to quantify sympathetic/parasympathetic balance; however, in mice this relationship is not so clear (26, 29). Both sympathetic and vagal modulations of the heart have significant roles in the genesis of LF oscillations in mice (26). Cholinergic blockade attenuates LF of PI variability as does genetic enhancement of β-adrenergic input or treatment with dobutamine (26, 29, 52, 54). On the other hand, the opposite effect (an increase in HR LF power) was achieved by β1-adrenergic blockade with metoprolol (54). Nevertheless, the choice of mice in the present study was justified by the increasing reliance on the use of this species for physiological genomic studies.

In addition to studies of HRV, spectral analysis has been applied to spontaneous fluctuations of AP (26, 29, 52). In humans and rats, it is well established that slow rhythms of BP (Mayer waves) are modulated by sympathetic activity, which is physiologically under the control of the baroreceptor reflex (3, 42, 46). In mice, spectral analysis has revealed cumulative variability between 0.08 and 1.0 Hz, characterizing the LF range in this species (26). Peripheral autonomic blockade with prazosin, a specific α1-adrenoreceptor antagonist, abolished the LF peak of BP spectra in mice, whereas inhibition of endogenous nitric oxide (NO) formation consistently enhances BP variability in the LF range (26, 52). This latter result suggests that endogenous NO could function as a buffer for such fluctuations. It is noteworthy that cholinergic or β-adrenergic blockade seems to have little effect on BP variability and its spectral parameters (26).

Even considering the limitation of transferring data regarding HRV from mice to other species, the usefulness of spectral analytic methods is clearly evident in the present study. Continuous measurement of BP and HR failed to reveal any effect of PB, stress, or the combination in the steady-state values of

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Table 1. MAP, and respective variability in time and frequency domains under basal and treatment conditions

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<tr>
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<th>Stress</th>
<th>PB</th>
<th>PB/Stress</th>
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<tr>
<td><strong>MAP, mmHg</strong></td>
<td>103 ± 5</td>
<td>101 ± 4</td>
<td>96 ± 4</td>
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<tr>
<td><strong>SD, mmHg</strong></td>
<td>3.2 ± 0.5</td>
<td>2.9 ± 0.3</td>
<td>3.3 ± 0.5</td>
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<tr>
<td><strong>LF, mmHg</strong></td>
<td>2.8 ± 0.5</td>
<td>2.7 ± 0.3</td>
<td>3.0 ± 0.5</td>
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Data are expressed as means ± SE. LF, low-frequency power; HF, high-frequency power; PB, pyridostigmine.

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Fig. 5. Blood cholinesterase activities (in μmol/min−1·ml−1) during the basal period and 3 days after treatment. *P < 0.05 compared with basal. ChE, total ChE activity; AChE, acetylcholinesterase; BChE, butyrylcholinesterase.
these cardiovascular parameters. In contrast, spectral analysis showed that there were marked changes (up to 2-fold) not only in PI variability in the time and frequency domain but also in the baroreflex index, when PB was associated with stress. Several studies have shown that HR and AP variabilities in the LF range are associated to the autonomic modulation of the cardiovascular system, because these oscillations are generated in the CNS and transferred to the periphery through autonomic nerves (2, 3, 10, 27, 39). Even though the LF oscillations could be differentially transmitted to the heart and vessels, HR fluctuations should not be totally disconnected from AP fluctuations because the baroreflex (10, 11, 46). In this way, changes in BRS could strongly affect the power of the HR oscillations in the LF range (16), which could explain, at least in part, the results found when combining PB treatment and stress exposure.

Studies in humans also failed to show changes in baseline hemodynamic parameters after PB treatment; changes were evident only when PB was combined with mental stress (40). Thus it appears that the ChE effects on HR are somehow dependent on possible alterations in the autonomic drive to the heart induced by 3 days of shaker stress. This is supported by data that suggest that autonomic drive input is critical in the mediation of variability, because treatment with autonomic blockers reduced LF variance and attenuated the response to stress (15).

The mechanism responsible for the enhanced PI variability and baroreflex index found in the PB/Stress group could involve afferent baroreflex input, autonomic drive to the circulatory system, or the complex integration of the baroreflex at the level of the CNS. Regarding the CNS, it is well known that the brain is protected from many classes of pharmacological substances by the BBB, including PB itself. This is not the case for the peripheral nervous system. Thus we would expect PB to manifest its pharmacological and toxicological actions primarily in the periphery. However, one cannot discount central actions because the BBB was modified in specific brain regions under stressful conditions (17, 48). The probable mechanism underlying BBB permeability in these conditions is speculative but may involve neurochemical mediators, such as serotonin (48). Infusion of this amine in small amounts increased BBB permeability within 15 min after administration. In another study, swim stress in mice increased BBB permeability, reducing the PB dose required to inhibit mouse brain AChE activity by 50% (17). This coincided with a >10-fold increase in brain penetration of an albumin-binding dye. Because we found the PB effect only in the presence of stress, it is reasonable to speculate that the effects observed are due to CNS interactions.

In line with other species, baroreflex control of HR seems to have an important role in the genesis of slow oscillations of HR in mice (26, 29). Therefore, it is not surprising that the marked increase in the power of LF oscillations of PI is associated with an increase in the α-index, a measure of spontaneous baroreflex. In humans, there was a relationship between vagal control of heart and BRS (22). Patients with low vagal tone had reduced BRS and vice versa for patients with high tone. Once again, the mechanism(s) responsible for the enhanced BRS associated with PB administration and stress could involve either of the mechanisms listed above (baroreflex afferent and effenter branches and the complex integration at the CNS).

In conclusion, spectral analysis of PI and BP variability revealed a hidden cardiovascular effect of the combination of PB treatment and stress exposure. Extended treatment with PB and stress resulted in dramatic changes in HRV and baroreflex function, likely associated with alterations in sympathetic/parasympathetic balance in the heart.

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