Acute and Repeated Restraint Stress Have Little Effect on Pyridostigmine Toxicity or Brain Regional Cholinesterase Inhibition in Rats

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Pyridostigmine, a carbamate cholinesterase (ChE) inhibitor, has been used for decades in the treatment of the autoimmune disorder myasthenia gravis and was used prophylactically to protect soldiers from possible organophosphorus nerve agent exposures during the Persian Gulf War. Pyridostigmine is a charged, quaternary compound and thus would not be expected to easily pass the blood-brain barrier. Some studies have suggested, however, that stress may alter blood-brain barrier integrity and allow pyridostigmine to enter the brain. We evaluated the effects of acute and repeated restraint stress on functional signs of cholinergic toxicity (i.e., autonomic dysfunction and involuntary movements) and brain regional cholinesterase inhibition following either acute or repeated pyridostigmine exposures. The acute, oral maximum-tolerated dosage (MTD) of pyridostigmine was estimated at 30 mg/kg. Peak ChE inhibition in whole blood occurred from 0.5 to 4 h after MTD exposure, whereas minimal (<20%) brain ChE inhibition was noted. For acute restraint studies, rats were either (1) restrained for 90 min and then given pyridostigmine (30 mg/kg, po), (2) given pyridostigmine and immediately restrained for 60 min, or (3) restrained for 3 h, given pyridostigmine, and restrained for an additional 60 min. In all cases, rats were evaluated for cholinergic toxicity (SLUD signs and involuntary movements) and sacrificed 1 h after pyridostigmine treatment. Plasma corticosterone was significantly elevated immediately after a single 60-min session of acute restraint stress, but returned to control levels by 1 and 3 h later. Pyridostigmine-induced toxicity was not enhanced nor was brain ChE inhibition altered by acute restraint stress. Blood-brain barrier permeability, assessed by accumulation of horseradish peroxidase in brain regions following intracardiac injection, was not increased by restraint stress. For repeated restraint studies, rats were given pyridostigmine (0, 3, or 10 mg/kg/day) immediately prior to daily restraint (60 min) for 14 consecutive days. Plasma corticosterone was elevated at 1 and 7 days but not at 14 days. Pyridostigmine-treated rats in both dosage groups exhibited slight signs of toxicity for the first 3–5 days, after which cholinergic signs dissipated. Repeated restraint had little effect on functional signs of pyridostigmine toxicity, however. Whole blood and diaphragm ChE were markedly reduced 1 h after the last treatment, but stress had no influence on ChE inhibition in either peripheral or central tissues. The results suggest that acute and repeated restraint stress have little effect on pyridostigmine neurotoxicity or apparent entry of pyridostigmine into the brain.

Key Words: pyridostigmine bromide; blood-brain barrier; immobilization; corticosterone; horseradish peroxidase; Gulf War illness.

Pyridostigmine, a carbamate cholinesterase (ChE) inhibitor, has been used clinically for decades to treat myasthenia gravis. Pyridostigmine is a polar chemical at physiological pH (containing a quaternary ammonium group) and therefore should be largely prevented from entry into the central nervous system by the blood-brain barrier. Generally, anti-ChE effects of pyridostigmine are considered to be limited to the peripheral nervous system (Taylor, 1990).

Pyridostigmine has a relatively short inhibitory action on ChE compared to organophosphorus inhibitors such as sarin, a nerve agent of concern in the Persian Gulf War. The temporary occupation (carbamyla tion) of the active site serine of acetylcholinesterase by pyridostigmine can prevent the long-term inactivation (phosphorylation) caused by nerve agents, therefore pyridostigmine was used prophylactically to protect soldiers from possible nerve agent exposures during the Gulf War (Keeler et al., 1991; Wenger et al., 1993).

After returning from the Gulf War, thousands of U.S. military personnel complained of a variety of symptoms, including prolonged fatigue, headaches, muscle and joint pain, sleep disturbances, cognitive difficulties, and others (Haley and Kurt, 1997; Joseph, 1997). In order to be effective, pyridostigmine was given to soldiers at relatively high dosages (i.e., sufficient to cause significant ChE inhibition). While pyridostigmine had been used safely for decades in the treatment of patients with myasthenia gravis, it could have different actions in persons without this disorder. Furthermore, a number of studies have suggested that certain environmental conditions may alter the neurotoxic effects of pyridostigmine. A critical study (Fried-
man et al. (1996) reported that forced swimming stress in mice disrupted blood-brain barrier integrity and was associated with marked increase in pyridostigmine penetration into the brain. A variety of other stress models have also been reported to increase blood-brain barrier permeability (Fatranska et al., 1987; Romero-Veccione et al., 1987; Sharma and Dey, 1986).

To test the hypothesis that stress modifies pyridostigmine neurotoxicity, we evaluated the effects of either acute or repeated restraint stress on pyridostigmine-mediated cholinergic toxicity and inhibition of ChE activity in central (frontal cortex, cerebellum, and hippocampus) and peripheral (whole blood and diaphragm) tissues. The results suggest that neither acute nor repeated restraint stress markedly influences the signs of neurotoxicity or ChE inhibition in central or peripheral tissues following pyridostigmine exposure.

**MATERIALS AND METHODS**

**Chemicals.** Pyridostigmine bromide (3-dimethylaminocarbonyloxy-N-methyl-pyridinium bromide), and peroxidase (Type II, from horseradish, activity 158 units/ml) were purchased from Sigma Chemical Company (St. Louis, MO). All chemicals were reagent grade.

**Animals and handling.** Sprague Dawley male rats were used throughout these studies. For acute restraint studies, 6-week-old rats were used. For repeated restraint studies, dosing started at 5 weeks of age and continued for 14 consecutive days. Animals were maintained and handled according to protocols outlined in the NIH/NRC Guide for the Care and Use of Laboratory Animals and reviewed by the Institutional Animal Care and Use Committee at Oklahoma State University. All rats were acclimated for 7 days before experimentation and allowed free access to food and water. Rats were kept on a 12-h light/dark cycle and isolated from environmental stressors (e.g., noise) as much as possible throughout the experiment. Pyridostigmine was prepared fresh each day in 0.9% saline (1 ml/kg, injection volume) and given to rats by oral gavage.

**Maximum tolerated dosage determination.** Rats (n = 6/treatment) were treated with 1 of 4 dosages of pyridostigmine (23, 30, 39, and 50 mg/kg) and observed for functional signs of toxicity and lethality for 24 h. Lethality was noted in rats treated with 39 mg/kg but not 30 mg/kg pyridostigmine, thus 30 mg/kg was defined as the maximum tolerated dosage (MTD).

**Stress protocols.** Rats were immobilized by placement in Plexiglas cylindrical restrainers (Model #51336, Stoelting Research Instruments, Wood Dale, IL). Three variations of the acute-restraint stress model were designed. Rats were either (1) placed in the restraint tubes for 90 min and immediately challenged with pyridostigmine, (2) treated with pyridostigmine immediately before placing them into the restraint tubes for 60 min, or (3) placed in the restraint tubes for 3 h, briefly removed, treated with pyridostigmine, and replaced into the restraint tubes for an additional 60 min. In all 3 cases, rats were given the MTD of pyridostigmine (30 mg/kg, po), observed for functional signs, and sacrificed for collection of tissues at 60 min after pyridostigmine dosing. For repeated restraint studies, model 2 above was utilized on a daily basis for 14 consecutive days, i.e., rats were given pyridostigmine (0, 3, or 10 mg/kg/day, po) and immediately placed in the restraint tubes for 60 min each day. In all acute and repeated restraint studies, rats were divided into 4 experimental groups with 6 rats per treatment group: (1) saline only (rats were kept in their home cage and treated with saline, 1 ml/kg, po), (2) pyridostigmine only (rats were kept in their home cage and treated with pyridostigmine in saline, 1 ml/kg, po), (3) restraint only (rats were immobilized in one of the above stress protocols and given saline), and (4) restraint + pyridostigmine (rats were immobilized and given pyridostigmine).

**Functional and behavioral measurements.** Animals were observed immediately before sacrifice (i.e., 60 min following pyridostigmine exposure) for involuntary movements and SLUD signs (acronym for salivation, lacrimation, urination, and defecation) by the methods of Moser and coworkers (1988) as described before (Liu and Pope, 1996). Involuntary movements were scored as 2 (normal quivering of vibrissae, head and limbs); 3 (mild, fine tremor typically seen in the forelimbs and head); 4 (whole body tremor); 5 (myoclonic jerks); and 6 (clonic convulsions). Autonomic dysfunction was scored as 1 (normal, no excessive secretion); 2 (slight, 1 SLUD sign or very mild multiple signs); 3 (moderate, multiple, overt SLUD signs); and 4 (severe, multiple, extensive SLUD signs).

**ChE activity evaluation.** Regional brain (frontal cortex, cerebellum, and hippocampus) and diaphragm samples were collected, dissected on ice, and washed with saline to remove contaminating blood. Blood samples were collected in Eppendorf tubes (1.5-ml) containing heparin (20 ml, 10,000 units/ml) immediately after decapitation. All tissues were stored at −70°C, thawed, and homogenized/diluted on the day of assay. Tissues were homogenized in 50 mM potassium phosphate buffer using a Polytron PT-3000 homogenizer (Brinkman Instruments, Westbury, NY) at 28,000 rpm for 20 s. ChE activity was measured radiometrically by the method of Johnson and Russell (1975) as previously described (Pope et al., 1991) using 1 mM [3H] acetylcholine iodide as the substrate. Incubation times and tissue concentrations required for linear rates of substrate hydrolysis were confirmed in preliminary assays. To minimize possible spontaneous reactivation of carbamylated ChE, conditions of limited tissue dilution and rapid assay following thawing and homogenization were adopted (Padilla and Hooper, 1992) as reported previously (Tian et al., 2002). Protein content was evaluated (Lowry et al., 1951) using 1 mg/ml bovine serum albumin as standard. Regional brain and diaphragm ChE activity was expressed as nmol/min/mg protein, whereas blood ChE activity was expressed as nmol/min/μl blood.

**Measurement of stress hormone.** For the acute restraint study, blood samples were collected (n = 6/treatment) immediately and 1 and 3 h following a single acute restraint session (60 min). For the repeated restraint study, blood samples were collected (n = 4/treatment) immediately after removal from the restraint tubes on days 1, 7, and 14. All procedures for collecting blood were completed within 1 min to minimize hormone secretion caused by handling stress. Blood in a heparinized Eppendorf tube was immediately centrifuged (10,000 rpm, 1 min at 4°C) to separate plasma, which was then frozen at −70°C until assay. Plasma corticosterone level was quantified using a radioimmunoassay kit (Rat Corticosteroid Coat-a-Count Kit, Diagnostic Products Corp., Los Angeles, CA).

**Horseradish peroxidase injection.** Measurement of HRP accumulation in the brain of untreated rats, following 60 min immobilization, was used to evaluate possible effects of acute restraint stress on BBB permeability. Immediately after being restrained for 60 min, rats were anesthesia with pentobarbital (50 mg/kg, ip). Ten min later, horseradish peroxidase (HRP) in saline containing 2% Evans Blue (100 mg/2 ml) was injected directly into the heart (2 ml/kg) by cardiac puncture over a 30-s period. Blue discoloration of the skin, eyes, and tail within about 1 min after the injection was regarded as a positive indicator of systemic injection and only those rats were used for subsequent analyses. Rats were decapitated 10 min after HRP injection. Whole brain was removed and rinsed with saline. Frontal cortex, cerebellum, and hippocampus were dissected and stored at −70°C until assay.

**HRP assay.** HRP activity was measured essentially by the method of Stewart and coworkers (1992), as reported previously (Tian et al., 2002). Regional brain tissues were homogenized in 50 mM sodium acetate buffer (pH = 5.0) using a Polytron PT-3000 homogenizer for 20 s at 28,000 rpm. Samples (0.5 ml) were transferred to Eppendorf tubes (1.5-ml) and incubated with 0.1 ml 1% Triton X-100 in sodium acetate buffer for 20 min. Samples were vortexed twice during the 20-min incubation and centrifuged (4 min, 12,500 rpm, 4°C) following incubation. Reaction mixtures contained 1 ml substrate (10 mg O-dianisidine HCl + 0.5 ml deionized water + 49 ml 50-mM acetate buffer + 0.5 ml 100-mM EDTA), 16.6 μl tissue sample, and 20 μl 0.3% H₂O₂. Absorbance at 460 nm was determined every 30 s after the start of reaction, for 2 min. HRP activity was expressed as ng HRP/mg tissue wet weight.
Statistical analysis. ChE activity was analyzed as nmol/min/mg protein and tested for significance by 1-way or 2-way analyses of variance (ANOVA), and HRP activity was tested by 1-way ANOVA, followed by linear contrasts, using the JMP statistical package (SAS, 1995). Plasma corticosterone levels were also tested by 2-way ANOVA. Functional signs of toxicity were reported as median/interquartile range (IQR) and tested for significance by the Pearson Chi-square test. Probability level less than 0.05 was considered statistically significant.

RESULTS

Maximum tolerated dosage (MTD) and cholinesterase inhibition following pyridostigmine exposure. The highest dosage of pyridostigmine tested that did not elicit any lethality, 30 mg/kg, was defined as the MTD. Figure 1A illustrates inhibition of whole blood ChE activity from 0.5 to 24 h following exposure to pyridostigmine (30 mg/kg, po in saline) and sacrificed 0.5, 1, 2, 4, 8, or 24 h following exposure for measurement of cholinesterase activity as described in Materials and Methods. (B) Rats (n = 6/treatment group) were treated with pyridostigmine (0.1, 0.3, 1, 3, 10, or 30 mg/kg, po) and sacrificed 1 h following exposure for measurement of cholinesterase activity. *Significant difference (p < 0.05) relative to control.

Corticosterone levels. Figure 2A shows plasma corticosterone in rats following 60 min of restraint stress. Plasma corticosterone was increased significantly (greater than 6 times of control) immediately after termination of restraint and returned to normal 1 h later. Figure 2B shows plasma corticosterone levels in rats following repeated, daily restraint stress. Corticosterone was significantly elevated immediately after termination of restraint on days 1 (about 7-fold) and 7 (about 4-fold), but not increased on day 14 of the study. These results suggest that both acute and repeated restraint models induced a significant stress response, and that tolerance to immobilization may eventually develop with daily restraint stress.

ChE activity. Table 1 shows the effects of acute or repeated pyridostigmine exposures on ChE activity in central (frontal cortex, cerebellum, and hippocampus) and peripheral (blood and diaphragm) tissues in the acute and repeated stress models. In general, pyridostigmine caused substantial inhibi-
tion of ChE activity in peripheral tissues, while having minimal and inconsistent effects on brain regional ChE activity. Acute pyridostigmine exposure in the acute restraint study caused substantial inhibition on whole blood ChE activity (86–95%). Daily pyridostigmine exposure (3 or 10 mg/kg) for 14 days caused marked inhibition (52–85%) in peripheral tissues (whole blood and diaphragm) but little change in brain regional ChE activity. None of the acute or repeated restraint procedures increased ChE inhibition by pyridostigmine in either peripheral or central tissues, however.

**Blood-brain barrier permeability following restraint stress.** Horseradish peroxidase accumulation in the brain following systemic administration was used to evaluate the possible effects of 60-min restraint stress on blood-brain barrier permeability. Figure 3 shows that HRP activity was not increased in frontal cortex, cerebellum or hippocampus by prior acute restraint stress.

**Functional toxicity measurements.** Figure 4 shows the effects of acute restraint stress on functional signs of cholinergic toxicity (SLUD and involuntary movements) following pyridostigmine exposure (30 mg/kg, po). In all cases, rats exposed to pyridostigmine showed only minimal signs of cholinergic toxicity (SLUD signs ranged from 2 to 2.5; involuntary movements ranged from 3 to 3.25). None of the stress models, however, influenced pyridostigmine-induced toxicity. Figure 5 shows the effects of daily restraint stress (60 min) on functional

### TABLE 1

**Influence of Restraint Stress on Cholinesterase Inhibition following Pyridostigmine Exposure in Rats**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Treatment</th>
<th>Frontal cortex</th>
<th>Cerebellum</th>
<th>Hippocampus</th>
<th>Blood</th>
<th>Diaphragm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Restraint-Trt</td>
<td>Con-sal</td>
<td>100.0 ± 6.5</td>
<td>100.0 ± 4.7</td>
<td>100.0 ± 2.5</td>
<td>100.0 ± 3.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Con-PYR</td>
<td>90.6 ± 10.8</td>
<td>99.6 ± 9.1</td>
<td>93.6 ± 5.0</td>
<td>9.5 ± 1.2*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Str-sal</td>
<td>93.6 ± 9.4</td>
<td>99.9 ± 4.4</td>
<td>91.4 ± 4.4</td>
<td>84.1 ± 7.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Str-PYR</td>
<td>103.2 ± 5.1</td>
<td>83.0 ± 4.5</td>
<td>101.6 ± 5.5</td>
<td>7.7 ± 0.7*</td>
<td></td>
</tr>
<tr>
<td>Restraint/Trt</td>
<td>Con-sal</td>
<td>100 ± 3.3</td>
<td>100.0 ± 6.4</td>
<td>100 ± 2.3</td>
<td>100.0 ± 5.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Con-PYR</td>
<td>79.8 ± 4.7*</td>
<td>97.8 ± 7.6</td>
<td>86.2 ± 5.7</td>
<td>13.8 ± 1.1*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Str-sal</td>
<td>106.9 ± 4.3</td>
<td>106.4 ± 6.4</td>
<td>93.8 ± 5.1</td>
<td>102.9 ± 5.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Str-PYR</td>
<td>78.6 ± 8.1*</td>
<td>88.3 ± 2.5</td>
<td>93.0 ± 6.8</td>
<td>12.8 ± 0.6*</td>
<td></td>
</tr>
<tr>
<td>Repeated Restraint (3 mg/kg/d)</td>
<td>Con-sal</td>
<td>100.0 ± 6.4</td>
<td>100.0 ± 3.0</td>
<td>100.0 ± 9.9</td>
<td>100.0 ± 13.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Con-PYR</td>
<td>76.5 ± 7.4</td>
<td>88.8 ± 5.0*</td>
<td>108.7 ± 3.0</td>
<td>4.9 ± 0.7*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Str-sal</td>
<td>96.7 ± 5.1</td>
<td>111.4 ± 5.5</td>
<td>110.1 ± 7.4</td>
<td>96.5 ± 8.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Str-PYR</td>
<td>81.0 ± 11.1</td>
<td>78.3 ± 3.4*</td>
<td>101.4 ± 4.0</td>
<td>8.3 ± 0.5*</td>
<td></td>
</tr>
<tr>
<td>Repeated Restraint (10 mg/kg/d)</td>
<td>Con-sal</td>
<td>100.0 ± 6.4</td>
<td>100.0 ± 2.7</td>
<td>100.0 ± 3.2</td>
<td>100.0 ± 6.6</td>
<td>100.0 ± 4.7</td>
</tr>
<tr>
<td></td>
<td>Con-PYR</td>
<td>93.1 ± 9.8</td>
<td>101.4 ± 2.6</td>
<td>106.5 ± 12.1</td>
<td>46.6 ± 3.3*</td>
<td>23.3 ± 1.6*</td>
</tr>
<tr>
<td></td>
<td>Str-sal</td>
<td>110.3 ± 4.7</td>
<td>103.3 ± 2.1</td>
<td>99.3 ± 9.2</td>
<td>110.9 ± 6.6</td>
<td>96.9 ± 6.1</td>
</tr>
<tr>
<td></td>
<td>Str-PYR</td>
<td>111.0 ± 7.0</td>
<td>106.2 ± 4.6</td>
<td>98.4 ± 2.8</td>
<td>46.5 ± 3.2*</td>
<td>17.7 ± 4.6*</td>
</tr>
</tbody>
</table>

**Note.** For each experiment, 24 rats were divided into 4 groups with 6 rats per treatment: Rats were either not stressed (Con) or restraint-stressed (Str) and either given saline (sal) or pyridostigmine (PYR). ChE activity was measured by the radiometric method as described in Methods. Whole blood ChE activity was calculated as nmol/min/μl blood (combined control value: 0.48 ± 0.09) whereas regional brain and diaphragm ChE activity was calculated as nmol/min/mg protein (combined control value: frontal cortex, 39.2 ± 7.2; cerebellum, 20.6 ± 3.8; hippocampus, 24.2 ± 4.4; diaphragm, 4.3 ± 1.2). ChE activity was expressed as mean percentage of nonstressed, saline control group (± SEM).

*Rats were restrained for 90 min, removed from restraint tubes and given pyridostigmine (30 mg/kg), and then sacrificed 1 h later for cholinesterase measurements.

†Rats were given pyridostigmine (30 mg/kg), immediately placed in the restraint tubes and then sacrificed 1 h later for cholinesterase measurements.

‡Rats were placed in restraint tubes for 3 h, removed and given pyridostigmine (30 mg/kg), replaced back into the restraint tubes for an additional 1 h and then sacrificed for cholinesterase measurements.

§Rats were given pyridostigmine (3 mg/kg/day) and placed in the restraint tubes for 1 h each day for 14 consecutive days. On the last day, rats were sacrificed immediately upon removal from the restraint tubes for cholinesterase measurements.

¶Rats were given pyridostigmine (10 mg/kg/day) and placed in the restraint tubes for 1 h each day for 14 consecutive days. On the last day, rats were sacrificed immediately upon removal from the restraint tubes for cholinesterase measurements.

*Significant difference from Con-sal group.
signs of toxicity in rats dosed daily with pyridostigmine (0, 3, or 10 mg/kg, po) for 14 days. Both pyridostigmine dosing groups (3 and 10 mg/kg) initially exhibited SLUD signs and involuntary movements in the first 3 to 5 days of dosing, after which no functional signs were noted. Again, however, repeated restraint stress had essentially no effect on pyridostigmine toxicity.

**DISCUSSION**

The hypothesis that stress-induced changes in permeability of the blood-brain barrier allowed pyridostigmine to enter the central nervous system and led to persistent neurological signs and symptoms in some Gulf War veterans has attracted considerable attention (Charatan, 1999; Grauer et al, 2000; Haley et al, 1997; Hanin, 1996; Sinton et al., 2000). In the present study, we evaluated the effects of either acute or repeated restraint stress on signs of cholinergic toxicity and comparative ChE inhibition in brain regions and peripheral tissues following pyridostigmine exposure in rats. Acute restraint and repeated restraint models elicited substantial elevation of plasma corticosterone, indicating the general validity of the stress models utilized. However, under a variety of stress-pyridostigmine interactive conditions (e.g., simultaneous stress and pyridostigmine vs. stress, followed by pyridostigmine, short-term vs. long-term stress, acute stress/acute pyridostigmine vs. repeated stress/repeated pyridostigmine), we found no evidence of stress-induced increased toxicity following pyridostigmine exposure. The results suggest that neither acute nor repeated restraint stress enhance ChE inhibition or cholinergic toxicity following high pyridostigmine exposures. While the dosages of pyridostigmine utilized caused substantial ChE inhibition in peripheral tissues (i.e., diaphragm, whole blood), pyridostigmine had little direct anticholinesterase action in the brain. We did, on occasion, note a slight reduction (11–22%) in ChE activity in some brain regions in some studies, e.g., frontal cortex of rats simultaneously restrained and given pyridostigmine (see Table 1). Although statistically significant, this degree of reduction in ChE activity would generally be considered to have little functional consequence. More importantly, however, in those cases where brain regional ChE inhibition...
was noted, the degree of inhibition was not influenced by restraint stress.

Horseradish peroxidase (HRP) accumulation in brain regions following systemic administration was used to evaluate possible blood-brain barrier permeability changes following restraint stress. While increased plasma corticosterone levels following restraint stress suggested the procedures were indeed stressful to the animals, we saw no evidence of HRP accumulation following a single session of 60-min restraint stress. We previously obtained very similar findings in both forced swimming and forced running stress models (Tian et al., 2002).

Together, these results suggest that 3 standard stressors in rodents have little effect on blood-brain barrier permeability changes to this protein tracer. While HRP and pyridostigmine are markedly different in size and thus their passage through the blood-brain barrier may be affected differently by permeability changes, the results indicating both a lack of HRP accumulation in the brain and no enhancement of pyridostigmine-mediated brain ChE inhibition by any stress model indicate that blood-brain barrier permeability was not influenced by restraint stress in our studies.

Friedman and coworkers (1996) first reported a substantial effect of forced swimming stress on pyridostigmine effects in the brain of mice, showing that the anticholinesterase potency of pyridostigmine was increased more than 100-fold by brief swimming stress episodes. Other indicators of enhanced entry of pyridostigmine into the brain (e.g., increased brain levels of c-fos and AChE mRNA) were also reported. The discrepancies between our findings and those of Friedman and colleagues (1996) regarding ChE inhibition could arise from a variety of factors, e.g., animal species, conditions of stress, etc. The age of the animal (and thus the maturational integrity of the blood-brain barrier) has also been noted as an important factor in sensitivity to possible blood-brain barrier disruption by environmental factors (Ben-Nathan et al., 1989; Sharma and Dey, 1986). We used young rats (5–7 weeks of age) in our studies, an age group previously shown sensitive to BBB disruption with environmental stressors (Sharma and Dey, 1986). It should be noted, however, that a number of more recent studies have also been unable to confirm the finding of stress-mediated enhancement of brain ChE inhibition by pyridostigmine. In guinea pigs exposed to extreme heat stress, pyridostigmine did not penetrate the brain (Lallement et al., 1998). The amount of 11C-labeled pyridostigmine in the brain following intravenous administration was not increased in mice following forced swimming (Telang et al., 1999). Grauer and coworkers (2000) reported that swimming stress or cold stress did not increase pyridostigmine-mediated inhibition of brain cholinesterase activity in mice. Sinton and colleagues (2000) actually noted reduced brain ChE inhibition by pyridostigmine following immobilization, forced swimming, or combined stress conditions. Ovadia and coworkers (2001) reported that a variety of stress models had little effect on blood-brain barrier permeability to Evans Blue dye. Long-term (3 days) intermittent foot-shock stress was reported to elevate plasma stress hormone levels but have no effect on pyridostigmine entry into the brain of rats (Kant et al., 2001). Our laboratory recently reported (Tian et al., 2002) a general lack of effect of either forced swimming or forced running on acute pyridostigmine-induced functional toxicity and ChE inhibition in brain and...
blood. Thus, a number of recent studies suggest that stress generally has little influence on the ability of systemic pyridostigmine to interact directly with brain acetylcholinesterase. While stress may not generally increase the entry of pyridostigmine into the brain, this does not rule out the possibility that peripheral actions of pyridostigmine could be modified by environmental stressors. Our results suggest that restraint stress has little effect on overt functional signs of cholinergic toxicity following high dosages of pyridostigmine. It is possible that stress could influence responses to low-dose pyridostigmine exposures, however. Nobrega and colleagues (1999) reported that comparatively low pyridostigmine exposure (45-mg dose) reduced the heart rate increase associated with a mental stressor (arithmetic test) in healthy human volunteers. In another study, this same group (Nobrega et al., 2001) reported that repeated pyridostigmine dosing (30 mg every 8 h) reduced heart rate and increased heart rate variability in healthy human volunteers over a 24-h period. Plasma cholinesterase inhibition in this study was reported to be \( \Delta \xi \% \) at 2 and 24 h after initiation of dosing. Thus, more subtle peripheral effects may occur with very low dose pyridostigmine exposures associated with negligible inhibition of blood cholinesterases. While blood cholinesterases are generally equally or more sensitive than cholinesterases in “target” tissue, acetylcholinesterase in some autonomic pathways regulating cardiac function could possibly be more sensitive and inhibited by lower dosages of pyridostigmine. It may also be that another target macromolecule aside from acetylcholinesterase, with higher sensitivity to pyridostigmine, is responsible for the reported changes in cardiac function. Pyridostigmine has been shown to interact directly with nicotinic (Akaeki et al., 1984; Albuquerque et al., 1988) and muscarinic (Lockhart et al., 2001) receptors, and such interactions could potentially be important in the observed modulation of cardiac function. Modulation of pyridostigmine-mediated chronicotrophic and heart rate variability changes by environmental stressors should be investigated.

Recently, Li and coworkers (2000, 2001) reported apoptosis in some brain regions following repeated pyridostigmine exposures in rats (1.85 mg/kg, ip, twice daily for 4 days). While pyridostigmine significantly reduced plasma ChE activity under these conditions, brain ChE was not significantly affected. Additional studies showed that apoptosis occurred in both cortical and cerebellar granule cells exposed in vitro to relatively high concentrations (10−250 mM, 24 h) of pyridostigmine. Pyridostigmine may induce apoptosis in neuronal cells in vitro, but the induction of apoptosis in brain by pyridostigmine in vivo (Li et al., 2000) could potentially occur through an indirect mechanism elicited by peripheral actions of the drug. If no significant brain AChE inhibition was noted under conditions where apoptosis occurred, either peripheral actions of the drug led to indirect changes in neuronal function within the brain or another neuronal target was responsible for initiation of these changes. Additional studies should further characterize mechanisms of neuronal apoptosis by pyridostigmine.

A recent population-based case control study of self-reported exposures concluded that most illnesses in Gulf War veterans could not be explained by exposures to agents which elicit toxicity through acetylcholinesterase inhibition (Spencer et al., 2001). The results from our study as well as a number of other recent reports indicate that stress does not generally increase the anticholinesterase actions of pyridostigmine in the central nervous system. While stress may in some way alter the toxicity of pyridostigmine under some conditions, the overall data available to date do not support a link between stress and enhanced cholinesterase inhibition/cholinergic toxicity following pyridostigmine exposure in the etiology of Gulf War illnesses.

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