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ACUTE vs SUBCHRONIC PYRIDOSTIGMINE ADMINISTRATION: EFFECTS
ON THE ANTICHOLINERGIC PROPERTIES OF ATROPINE

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ABSTRACT

Acute, subchronic and chronic exposures to cholinergic compounds may result in differing effects. The efficacy of pyridostigmine bromide (PY) prophylaxis against organophosphorus poisoning depends on post exposure atropine (AT) administration. AT induces a dose-dependent increase in rate of rise of core temperature in heat exposed humans and rats. To determine whether AT's anticholinergic potency is altered following PY administration, we examined AT's effects following acute or subchronic (2 weeks) PY administration in the sedentary heat-stressed rat. Unrestrained rats were used in the following 8 groups of 12: acute (a, 2 injections via tail vein) aSAL+SAL, aSAL+AT, aPY+SAL, aPY+AT; subchronic (c, osmotic pump + tail vein) cSAL+SAL, cSAL+AT, cPY+SAL, cPY+AT (SAL- saline, AT- 200 ug/kg, aPY- 100 ug/kg, cPY- 20 ug/hr.) Fifteen minutes following the final injection, rats were subjected to an ambient temperature of 41.5°C until a core temperature of 42.6°C was attained. Heat tolerance times were significantly improved for cPY+SAL over aPY+SAL (241 ± 9 vs 187 ± 16 min, mean ± SE) and for cPY+AT over aPY+AT (76 ± 9 vs 57 ± 2 min). The improvement in thermoregulation resulted from increased salivary water for evaporative cooling indicated by % weight loss (corrected for fecal loss) during heat stress: cPY+SAL over aPY+SAL (8.4 ± 0.3 vs 6.6 ± 0.5 %) and cPY+AT over aPY+AT (2.1 ± 0.4 vs 1.8 ± 0.2 %).

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INTRODUCTION

Acute, subchronic (administration for a time span of a few days to 10% of the life span of the animal) and chronic exposures to cholinergic compounds have resulted in differing effects (6,9,27). Acute pyridostigmine (PY) was found to elicit ultrastructural alterations at the neuromuscular junction of rat diaphragm muscle (3,12) similar to those seen with acute physostigmine (PH) administration (20). These alterations were reversed with subchronic administration (3,20). Acute, but not subchronic, administration of the PH resulted in decreased endurance in a running rat model (20,21,24). Several, but not all, of the anticholinesterase soman's behavioral, enzymatic and physiological effects were reversed upon repeated sublethal dosing (27). Since the doctrinal employment of PY as a pretreatment involves sustained use (30 mg. tablets t.i.d. for 2 weeks), the potential for adverse side effects in healthy individuals should be examined in both subchronic and acute dosing regimens.

Pyridostigmine has been used as a prophylaxis against organophosphorus poisoning (14,4). The efficacy of PY for this purpose depends on the administration of atropine (AT) after exposure (2,9). However, AT suppresses sweating in man thus resulting in reduced evaporative cooling during passive exposure to hot environments or during exercise even in moderate environments (7,17). In hot environments, rats spread saliva over the ventral surface of their bodies for evaporative cooling (10). Both saliva secretion in rodents (11,23) and sweat secretion in humans (15,16) are inhibited by AT. Clubleby *et al.* (5) simultaneously measured sweating and salivation in humans and determined that they were similarly inhibited by AT.

We previously reported that AT induces a dose-dependent increase in heating rate (rate of rise of core temperature) in the sedentary heat-stressed rat (11,23). This is a muscarinic anticholinergic effect due to inhibition of saliva secretion. A 2 mg dose of AT will suppress the sweat rate of heat-exposed humans by about 40% (7,26), also a muscarinic effect. In the rat, 200 ug/kg is equivalent to the standard 2 mg dosage in man (8); fortuitously, 200 ug/kg of AT elicits a similar 40% inhibition of salivary water loss in the rat (11,23). Additionally, the time course of action of AT in the rat closely approximates that reported in humans (23).

PY has been used clinically to counter the anticholinergic effects of AT, and AT to counter the anticholinesterase properties of PY (9,14). Anticholinesterase administration will stimulate salivation or sweating independent of heat exposure, but the doses required are ordinarily higher than those eliciting 40% cholinesterase (ChE) inhibition (9,31). Because PY may neutralize the muscarinic anticholinergic properties of AT, it may be necessary to give larger therapeutic doses of AT for OP poisoning if PY has been given prophylactically (14,22). However, the anticholinergic potency of AT administered following

PY may vary depending on whether the PY was given acutely or subchronically.

The phenomenon of cholinergic tolerance typically is accompanied by reduced sensitivity to agonists and increased sensitivity to antagonists (6). If supersensitivity to antagonists accompanies development of tolerance after subchronic PY administration, an increased anticholinergic response to AT might be expected. To determine if there is an alteration of AT's anticholinergic properties following subchronic PY administration, we examined the effects of AT administration following acute or subchronic PY administration in the sedentary heat-stressed rat.

Oral administration of the recommended prophylactic dose of PY (3 - 30mg tablets/day) resulted in 30-40% inhibition of ChE in human subjects (13,17,28). Therefore, it was necessary to determine the doses of acute and subchronic PY that elicited 40% ChE inhibition in rats prior to examining the effects of acute and subchronic PY administration on the anticholinergic potency of AT administration in sedentary heat-stressed rats.

METHODS

Animals

Adult male Sprague-Dawley rats (Charles River, CD strain, 510-530g) were caged individually in wire-bottomed cages in an environmental chamber (4 x 3 x 2 m) at 26°C and 50% rh, and used one time only. Lighting was controlled automatically (on, 0600-1800 h) and Purina rat chow and water were available ad lib. except during experimental intervals.

Drugs

The form of atropine used was atropine alkaloid (Atropen[®] Injection Solution, Survival Technology, Inc., Bethesda, MD, Lot# VU5324). This solution was diluted with sufficient saline to provide a 200 ug/kg dose of AT in 0.2 ml for tail vein injection. The form of PY was pyridostigmine bromide (ICN Biochemicals, Cleveland, OH, Lot# 32274). For the acute experiments, PY was dissolved in 0.2 ml of sterile 0.9% saline and administered via lateral tail vein (iv). In the subchronic experiments, PY was administered (5 ul/hr) via osmotic mini-pump (model 2ML2, Alza, Palo Alto, CA). Osmotic pumps (Alzet model 2ml-2) were implanted subcutaneously under methoxyflurane anesthesia according to the procedure outlined by the manufacturer.

Cholinesterase (ChE) inhibition

To approximate acute and subchronic doses of PY to elicit a 40% inhibition of whole blood ChE, a range of doses were used with 3 rats per dose. Once appropriate doses were approximated, numbers of animals in each of 4 groups (saline iv, PY iv, saline via osmotic pump, PY via osmotic pump) were increased to 8.

To determine % ChE inhibition in the acute studies, a blood sample (0.3 ml) was drawn prior to PY injection and again one hour post injection. In the subchronic experiments, a blood

sample was drawn from the tail vein prior to osmotic pump implantation and additional 0.3 ml samples were drawn 1, 3, 7, 10, and 14 days after implantation. All samples were analyzed for whole blood ChE activity using our modification (19) of the Boehringer Mannheim Diagnostics' ReagentSet Cholinesterase #124117.

Heat stress procedure

Eight groups of 12 rats were used as follows:

Acute (a) studies - 2 (0.2 ml) tail vein injections 15 min apart-
aSAL (saline) + SAL
aSAL + AT (200 ug/kg)
aPY (100 ug/kg) + SAL
aPY + AT

Subchronic (c) studies- PY or saline via osmotic pump for 2 weeks and 1 (0.2 ml) tail vein injection-
cSAL + SAL iv
cSAL + AT iv
cPY (20 ug/hr) + SAL iv
cPY + AT iv

Fifteen min after the last iv injection the rats were heat stressed unrestrained in their own cages. They were placed in a 1 x 2 x 2 m chamber maintained at 41.5°C and 30% rh until a core temperature (Tc) of 42.6°C was attained. Tc (rectal probe inserted 6.5 cm into the rectum) was monitored every 15 min or sooner as Tc = 42.6°C was approached, and the animals were weighed every 30 min. Upon attaining a Tc of 42.6°C, the animals were removed from the heat, weighed, and allowed to cool passively in a 26°C chamber with core temperature monitored continuously until the core temperature returned to 40.4°C. During heating, weight loss (as a measure of saliva or urine spread for evaporative cooling) corrected for fecal pellet production, core temperature, and the extent of saliva or urine spread (23) were monitored.

Statistical analysis: Data were analyzed by an unpaired "t" test for comparisons between acute and subchronic groups with the same drugs; all other data comparisons (mean $\pm$ SE) were accomplished by a one-way analysis of variance followed by the Student-Newman-Keuls multiple range test for all pair comparisons. The null hypothesis was rejected at the $p < 0.05$ level.

RESULTS

In the acute studies, % ChE inhibition was determined to be $6 \pm 6\%$ after saline administration and $44 \pm 2\%$ 1 hr following administration of 100 ug/kg of PY. Two weeks following osmotic

pump implantation in the subchronic studies, % ChE inhibition was $3 \pm 8\%$ with saline and $48 \pm 4\%$ with 20 ug/hr of PY.

The 8 groups were divided into the 4 receiving no AT and the 4 receiving AT in Table I. AT significantly reduced the rate of water loss, the endurance time, and fecal loss while increasing the rate of rise of core temperature during the heat stress.

TABLE I

Comparison of Heat Stress Data with and without Atropine

GROUPS (N=48)	Wt loss (g/min)	Heat rate ¹ (°C/min)	Heat time (min)	Fecal loss (g)
No AT ²	0.18 ± 0.01	0.021 ± 0.001	215 ± 8	1.8 ± 0.2
With AT ³	0.14* ± 0.01	0.078* ± 0.002	59* ± 3	0.2* ± 0.1

Values are mean ± SE.

1 Rate of rise of core temperature.

2 All groups without atropine combined.

3 All groups with atropine combined.

* Significantly different from No AT group

The groups (Fig. 1) receiving AT lost significantly less water (weight loss corrected for fecal pellet production) than those with no AT, and there was a significant increase in the % weight loss of the group receiving cPY + SAL as compared to the group receiving aPY + SAL.

The extent of saliva spread was scored from 0 (no spread) to 3 (entire ventral surface covered). The groups (Fig. 2) not receiving AT all spread saliva maximally. The groups with both PY and AT spread to a greater extent than those with AT alone, and the cPY + AT groups spread to a greater extent than the aPY + AT group. Because of the somewhat subjective nature of this measurement, no statistics were done on the extent of saliva spread data.

The data represented in Fig. 3 indicate that the groups receiving cPY had lower heating rates than those with aPY although the difference was significant only in the PY + SAL groups. Both the acute and subchronic SAL + AT groups had significantly increased heating rates (Fig 3) and decreased endurance times (Fig 4) compared to the corresponding PY + AT groups. Fig. 4 indicates that both groups with cPY had significantly longer endurance times (to reach an T_c of 42.6°C) in the heat than the aPY groups.

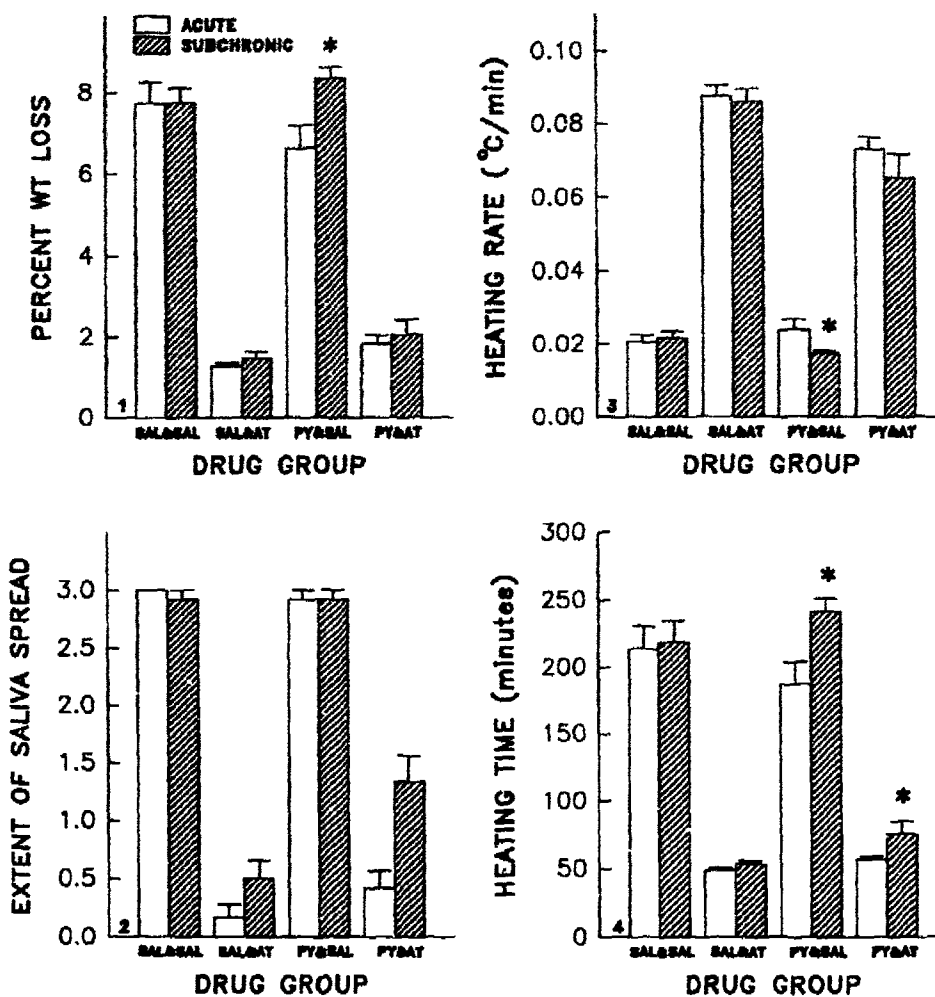


Fig. 1- % weight loss corrected for fecal pellet production; Fig. 2- Extent of saliva spread scored from 0 (no spread) to 3 (entire ventral surface covered); Fig. 3- Heating rate (rate of rise of core temperature); and Fig. 4- Heat time (to reach a core temperature of 42.6°C) during the heat stress for acute vs subchronic groups. Groups are as listed in Methods; values are mean $\pm$ SE; * indicates a significant difference between acute and subchronic groups of the same drug group.

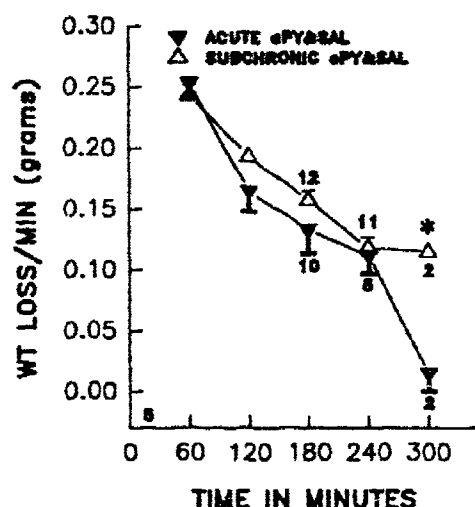


Fig. 5- Weight loss/min for each hour of heat stress for aPY + SAL and cPY + SAL groups. * indicates a significant difference acute vs subchronic groups. The numbers reflect the animals remaining in the heat out of 12.

Weight loss per min for each hour of the heat stress is plotted in Fig. 5 for the aPY + SAL and cPY + SAL groups. After the first hour, the cPY + SAL group had consistently higher water loss for evaporative cooling although the difference was significant only at 300 min. Of the 12 animals in each group, at 180 min 12/12 in the cPY group but only 10/12 in the aPY group were still in the heat (had not yet reached the end-point core temperature of 42.6°C). At 240 min 11/12 animals remained in the cPY group but only 5/12 in the aPY group. At 300 min 2/12 animals remained in each group, but the cPY animals had a significantly elevated water loss rate.

DISCUSSION

The 44% ChE inhibition induced by subchronic administration of 20 ug/hr of PY agrees with the 20 and 60% inhibition reported by Hudson *et al.* (12) for subchronic administration of 10 ug/hr and 60 ug/hr, respectively, of PY via osmotic pump. The 48% whole blood ChE inhibition resulting from acute administration of 100 ug/kg of PY in the present study was higher than the previously reported (25) 40% inhibition of serum ChE following 400 ug/kg. However, in this study pyridostigmine bromide salt was used, and the injectable drug Mestinon (Roche Laboratories) was used in the earlier study. Pyridostigmine is a quaternary amine and only about 1/30th of an oral dose is absorbed in the gut (9); given a 30 mg tablet, approximately 1 mg/70 kg man will be absorbed. If this 1 mg/70 kg is multiplied by 7 (mass to surface area conversion factor from man to rat (8)), this equates to the 100 ug/kg dose of PY used in this study.

Atropine elicited decreases in endurance time, wt loss rate, fecal loss, and extent of saliva spread as well as an increase in heating rate. Fecal loss was measured both to correct weight loss to more accurately reflect water loss and as an index of AT's inhibitory effect on intestinal motility. All of these effects are consistent with earlier reports from this laboratory (22,23,18), and the increased rate of rise of core temperature and decreased water loss rate are also consistent with reports of AT's effects in man (7,15,16,17) and primates (1). The administration of PY with AT attenuated the AT-induced increased heating rate and decreased water loss rate. In an earlier report (22) with the same dose of AT but 500 ug/kg of PY instead of the 100 ug/kg in the present study, the AT-induced decreases in water loss rate and increased heating rate were almost completely prevented; thus, these PY-induced effects are dose dependent.

Anticholinesterases have been associated with the development of tolerance upon repeated administration, and associated subsensitivity to agonists and supersensitivity to antagonists (6,29). Although these phenomena are most marked with organophosphorus anticholinesterases, evidence for supersensitivity to antagonists after carbamate administration have been noted (6).

The data in this study do not provide support for the hypothesis that subchronic administration of the carbamate PY would lead to supersensitivity to subsequent administration of the cholinolytic antagonist AT. Such a finding would have been of particular concern to military forces in a chemical warfare environment. The reduced tolerance to heat stress seen in atropinized individuals is normally compounded by wearing of Mission Oriented Protective Posture (MOPP) gear and the associated constraints on dissipation of metabolically-generated heat to the surrounding environment. Development of supersensitivity to atropine among individuals in MOPP also taking PY as a pretreatment for nerve agent intoxication could well generate cases of potentially fatal heat stroke due to accidental atropinization.

Among the more plausible explanations for the absence of tolerance or supersensitivity is that the level of cholinesterase inhibition achieved was insufficient to activate the mechanisms responsible for development of tolerance and supersensitivity. More intriguing is the possibility that any potential for demonstration of supersensitivity to the antagonist AT was masked by the induction of heat acclimation.

The augmented water loss rate and duration of water loss (Fig 5) seen with cPY compared to aPY resulted in increased heat tolerance (endurance time in the heat) and diminished heating rate. These changes are similar to some of the changes described as heat acclimation. Since increased sweat rate is required to produce satisfactory acclimation with greater sweat rates resulting in better acclimation (30), repeated bouts of exercise in the heat are usually required. Previous attempts to demonstrate pharmacologically-induced acclimation of sweat

glands have not been successful, but these have involved small areas of the body (30) and may not have resulted in sufficient stimulation of sweat glands. Since thermally-induced sweat in man and thermally-induced salivary water loss in rats are cholinergically regulated in a similar manner, subchronic administration of the anticholinesterase PY may have produced a physiological state similar to heat acclimation. However, several other indices of acclimation should be examined.

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