Multiple Exposures to Sarin Vapor Result in Parasympathetic Dysfunction in the Eye but not the Heart

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Several studies in conscious animals have reported parasympathetic dysfunction in the eyes following exposure to cholinesterase inhibitors. Given the similarities between the autonomic innervation in the eye and the heart, it is possible that parasympathetic dysfunction could also occur in the heart. Therefore, the present study assessed time domain indices of heart rate variability in conscious rats surgically implanted with telemetric transmitters to investigate the hypothesis that multiple exposures to the nerve agent sarin would result in muscarinic receptor desensitization and parasympathetic dysfunction in the heart. Animals exposed to sarin vapor on multiple occasions developed parasympathetic dysfunction in the eye characterized by an attenuated response to light and a diminished miotic response to sarin vapor exposure. However, the same dose of sarin vapor failed to produce any effects on either time domain indices of HRV or the magnitude of the tachycardia induced by atropine, suggesting that autonomic control in the heart was not affected. It is possible that the dose of sarin used in the present study was insufficient to inhibit cardiac acetylcholinesterase (AChE). Additional studies utilizing higher doses of sarin may be able to inhibit cardiac AChE, producing overstimulation of cardiac muscarinic receptors, ultimately resulting in desensitization and parasympathetic dysfunction.

Key Words: Sarin; organophosphate; heart rate variability; miosis; parasympathetic dysfunction.

The autonomic nervous system is responsible for the unconscious regulation of many organ systems. In the ocular system, both the sympathetic and parasympathetic branches of the autonomic nervous system play a role in the control of pupil size. Acetylcholine released by parasympathetic neurons binds to muscarinic receptors present on the rat pupillary sphincter, producing constriction of the pupil. Muscarinic antagonists produce dilation of the pupil, again demonstrating

the role for muscarinic receptors and the parasympathetic nervous system in the control of pupil size (Furuta *et al.*, 1998; Smith *et al.*, 1996). Norepinephrine released from sympathetic neurons binds to α-adrenergic receptors located on the radial muscles of the iris, resulting in dilation of the pupil (Yu and Koss, 2002, 2003).

O-isopropyl methylphosphonofluoridate, also known as sarin or by its military designation GB, is a highly toxic organophosphorous compound that produces the majority of its toxic effects through inhibition of the enzyme acetylcholinesterase (AChE). Inhibition of AChE results in the accumulation of the neurotransmitter acetylcholine and excessive stimulation of cholinergic receptors. In the eye, the overstimulation of muscarinic receptors on the pupillary sphincter muscle results in excessive contraction of the pupils, or miosis. Miosis occurs at concentrations of sarin many times lower than those required to cause death, and is one of the threshold clinical effects that can be observed following less-than-lethal exposures to sarin vapor (Mioduszewski, 2001; Mioduswewski *et al.*, 2002).

It has been demonstrated previously that repeated low-dose exposure to a cholinesterase inhibitor can result in dysfunction of the parasympathetic branch of the autonomic innervation of the eye in rats and guinea pigs (Dabisch *et al.*, 2005a,b; Soli *et al.*, 1980). This dysfunction appears to be due to a decrease in pupillary muscarinic receptor function, but not a decrease in receptor number (Dabisch *et al.*, 2005b; Soli *et al.*, 1980). Similarly, repeated topical ocular exposure of monkeys to the cholinesterase inhibitors echothiophate and isoflurophate resulted in a gradual decrease over time in the miotic response to both compounds, and a decreased miotic response to the cholinomimetic carbachol, again demonstrating the development of parasympathetic dysfunction (Bito and Banks, 1969).

In the heart, the autonomic nervous system regulates heart rate and contractility. The parasympathetic branch releases acetylcholine onto nodal muscarinic receptors resulting in a decrease in heart rate. Similar to muscarinic receptors located elsewhere in the body, it has been demonstrated that cardiac muscarinic receptor function is altered following overstimulation. In both beagle dogs and rats, multiple exposures to the

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cholinesterase inhibitor diisopropylfluorophosphate resulted in a 30–40% decrease in the number of cardiac muscarinic receptors (Vallette *et al.*, 1997; Zhu *et al.*, 1991). In cultured rat atrial cells and Chinese hamster ovary cells transfected with muscarinic receptors, incubation with the muscarinic agonist carbachol depressed the response of muscarinic receptors to acetylcholine by greater than 90% (Shui *et al.*, 2002).

In conscious animals, autonomic tone in the heart can be quantified by assessing heart rate variability (HRV). HRV is the variation in the interbeat (R-R) interval of the electrocardiogram (EKG) that is normally present due to slight changes in cardiac autonomic input. By examining several different measures of HRV, both the sympathetic and parasympathetic components of total autonomic tone can be quantified. This information has prognostic value in several pathological conditions. Decreased HRV correlates strongly with the incidence of mortality following acute myocardial infarction, as well as with the development of autonomic neuropathy in diabetics (Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). HRV has also been shown to be depressed in patients susceptible to sudden cardiac death (Singer *et al.*, 1988). Additionally, HRV can be used to assess the effect of drugs and chemicals on cardiac autonomic function. Administration of anticholinergic drugs, such as atropine, has been shown to decrease HRV, demonstrating the significant role of the parasympathetic nervous system in the regulation of heart rate (Pentilla *et al.*, 2005).

While the desensitization of cardiac muscarinic receptors following excessive cholinergic stimulation has been reported previously, there are no studies documenting the effect of repeated exposures to a cholinesterase inhibitor on autonomic control of the heart in conscious animals. However, several studies in conscious animals have reported parasympathetic dysfunction in the eyes following exposure to cholinesterase inhibitors. Given the similarities between the autonomic innervation in the eye and the heart, it is possible that parasympathetic dysfunction could also occur in the heart. Therefore, the present study assessed HRV in conscious rats surgically implanted with telemetric transmitters to investigate the hypothesis that multiple exposures to the nerve agent sarin would result in muscarinic receptor desensitization and parasympathetic dysfunction in the heart. If cardiac muscarinic receptors desensitize following repeated exposure to sarin vapor, it would be expected that HRV would be decreased in a manner similar to that seen following administration of an anticholinergic drug such as atropine since this situation mimics muscarinic receptor dysfunction.

MATERIALS AND METHODS

Animal use. Male Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) weighing 200–300 g were used in this study. All experiments and procedures were approved by the U.S. Army Edgewood Chemical Bio-logical Center Institutional Animal Care and Use Committee, and conducted in accordance with the requirements of U.S. Army Regulation 70-18 (Army Regulation 70-18) and the National Research Council's ''Guide for the Care and Use of Laboratory Animals'' (Institute of Laboratory Animal Resources, 1996).

Sarin vapor generation. Sarin vapor generation was accomplished as described previously (Muse *et al.*, 2006). Briefly, neat liquid sarin (Edgewood Chemical Biological Center, Aberdeen Proving Ground, MD) was placed into a gas tight syringe that was mounted on a variable rate syringe drive (Model 22, Harvard Apparatus Inc., South Natick, MA). The syringe drive delivered a constant flow of sarin through a flexible plastic line into a spray atomization system (Spray Atomization Nozzle ¼ J SS, Spraying Systems Co., Wheaton, IL). As liquid sarin entered the atomizer, compressed air (30–40 psi) entered through a side inlet and atomized the sarin liquid into fine droplets. Due to the volatility of sarin, these droplets quickly evaporated, and were drawn into a 750-l dynamic airflow inhalation chamber. The interior of the exposure chamber was maintained under negative pressure ($-0.50''$ H_2O), which was monitored with a calibrated magnehelix (Dwyer, Michigan City, IN). A thermo-anemometer (Model 8565, Alnor, Skokie, IL) was used to monitor chamber airflow at the chamber outlet. Sarin vapor from chamber air was collected onto a 10-mm Tenax TA—Hayesep D solid sorbent tube (CDS Analytical, Oxford, PA). Sarin concentrations were then determined using a gas chromatograph equipped with a flame ionization detector (Agilent Technologies, Inc., Wilmington, DE). The concentration of sarin vapor in the air that causes miosis in 50% of exposed male rats (EC_{50}) for a 60-min exposure is 0.030 mg/m^3 (Mioduszewski, 2002), while the concentration that results in death in 50% of exposed male rats (LC_{50}) for a 60-min exposure is 7.55 mg/m^3 (Mioduszewski *et al.*, 2002). The target sarin vapor concentration for each exposure in the study was 4.0 mg/m^3, above the EC_{50} for miosis, but below the LC_{50} for a 60-min exposure. This dose of sarin vapor has been shown previously to produce autonomic dysfunction in the eye without producing other serious toxic effects (Dabisch *et al.*, 2005a,b).

Exposure protocol. Rats were exposed to either sarin vapor or air in a whole-body dynamic flow inhalation chamber for 60 min on each of 3 consecutive days. Exposures occurred at 24-h intervals. As stated previously, the target sarin vapor concentration for each exposure in the study was 4.0 mg/m^3. Rats were euthanized 2 weeks following the final exposure. Ocular and cardiovascular measurements were made throughout the preexposure, exposure, and postexposure periods as described in the following sections.

Ocular measurements. The right eye of all rats in the study was digitally photographed in order to determine the degree of miosis produced by exposure to sarin vapor. This technique has been described previously (Dabisch *et al.*, 200b). Briefly, animals were temporarily hand restrained (< 20 s) to minimize movement of the head and the pupil was illuminated with an infrared spotlight (SL1236, Advanced Illumination, Rochester, VT). Several images were digitally recorded using an infrared capable video camera (model XC-ST50, Sony) equipped with a 75-mm/F2.7 lens (model LMV7527) and an image acquisition PC card (model PCI-1411, National Instruments Corp., Austin, TX). All images were taken under low-light conditions (< 5 lux). Pupil and iris radii were determined using custom image analysis software written using LabView 6 (National Instruments Corp., Austin, TX). For each animal, the pupil and iris measurements from two to three images were averaged. The results were expressed as the ratio of the pupil radius to the iris radius in order to correct for any variability between animals in the distance from the camera to the eye. Pupil size was measured 1 h before and 20 min after each exposure, and then 24, 48, and 72 h following the third exposure in order to determine the magnitude of the response elicited by sarin exposure, as well as estimate the rate of pupil recovery postexposure.

The pupillary light reflex in the right eye of all rats in the study was also measured in order to determine whether a given dose of sarin produced alterations in parasympathetic function. Similar techniques have been used previously (Dabisch *et al.*, 2005a). Briefly, animals were temporarily hand restrained (< 90 s) to minimize movement of the head and the pupil was illuminated with an infrared spotlight (model SL1236, Advanced Illumination,

Rochester, VT). A 300-lux light was directed onto the right eye of the animal for 2 s. Digital images of the eye were taken at a rate of 1/s until the pupil had returned to its baseline diameter. Pupil images were acquired and analyzed using the same acquisition and analysis system described previously. All light reflexes were recorded under controlled low-light conditions (< 5 lux), and only after the animals had been allowed to adjust to the darkness for at least 5 min. A light reflex was recorded 1 h before the first exposure, 1 h before the third exposure, and at 24, 48, 72, and 96 h following the third exposure.

Cardiac measurements. Telemetric transmitters (Model C50-PXT, Data Sciences, Inc., Minneapolis, MN) were surgically implanted to monitor the EKG. For the implantation of the telemetric probe animals were anesthetized with inhaled isofluorane (3.0% initially; 2.0% maintenance), and positioned on a heated surgical table. The hair on the ventral abdomen was clipped, and the area was scrubbed with chlorhexidine and isopropyl alcohol. Incisions were made along the midline of the abdomen, at the right side of the chest, and just below the left side of the ribcage. EKG leads were positioned subcutaneously at the right side of the chest and just below the left side of the ribcage in a lead II configuration, and sutured to the underlying muscle. The telemetric transmitter/ battery pack was inserted into the abdominal cavity and sutured to the abdominal muscle wall. The abdominal muscle incision was closed using nonabsorbable suture, and the skin incision was closed using staples. Prior to closure, the skin incisions were infiltrated with marcaine to provide postoperative analgesia. The depth of anesthesia was monitored throughout the surgical procedure by assuring that there is no response to tail or paw pinch. Animals were allowed to recover for at least 5 days prior to measurement of the EKG. Recordings of the EKG were made at 5000 Hz with a filter cutoff of 1250 Hz using DataQuest ART 3.01 software (Data Sciences, Inc., Minneapolis, MN). EKG data were imported into ECG-Auto software (Emka Technologies, Falls Church, VA) for analysis.

Time domain indices of HRV were calculated to determine the effect of sarin vapor exposure on autonomic tone in the heart. R-R intervals derived from the EKG were used to calculate (1) the standard deviation of the interbeat interval of normal beats (SDNN) and (2) the root of the mean of the squared differences between successive R-R intervals (rMSSD). SDNN is an indicator of total autonomic input to the heart, while rMSSD is an indicator of parasympathetic input to the heart (El-Mas and Abdel-Rahman, 2003; Sgoifo *et al.*, 1997; Stein *et al.*, 1994). In order to characterize the baseline autonomic tone in the rat heart, five control animals implanted with telemetric transmitters were treated with atropine (5 mg/kg im) to determine the effect of para-sympathetic blockade on time domain indices of HRV. Seven days later, the same animals were treated with propranolol (5 mg/kg im) to determine the effect of sympathetic blockade on time domain indices of HRV. The doses of atropine and propranolol used are similar to doses used previously to produce blockade of the parasympathetic and sympathetic branches of the autonomic nervous system, respectively (Towa *et al.*, 2004), and produced immediate changes in heart rate following administration relative to saline injected controls. Animals received saline injections on the 3 days before administration of either atropine or propranolol to determine the effect of the injection on HRV parameters. The EKGs of these animals were analyzed for changes in the time domain indices of HRV relative to saline injected control recordings.

Rats implanted with telemetric transmitters were exposed to either sarin vapor or air on each of 3 consecutive days as described previously. Animals were placed in exposure cages on each of the 3 days before exposure to sarin vapor in order to acclimate them to the cage. The EKGs from these animals were analyzed for time domain indices of HRV at the following time points: preexposure, 2 h following exposure #3, 24 h following exposure #3, 4 days following exposure #3, 7 days following exposure #3, and 14 days following exposure #3. Several rats exposed to sarin vapor also received injections of atropine (5 mg/kg im) 4-days preexposure, and at 24-h postexposure #3, 4-days postexposure #3, and 14-days postexposure #3 to determine the magnitude of the increase in heart rate produced.

Several additional rats without telemetric transmitters were also exposed so that AChE and butyrylcholinesterase (BChE) activities in whole blood could be measured. These assays are based on the Ellman *et al.* (1961) method and have

been described previously (Dabisch *et al.*, 2005b). Approximately 50 μl of blood was collected for use in these assays from a tail snip at the following time points: preexposure, 30 min following exposure #1, 60 min before exposure #2, 30 min following exposure #2, 60 min before exposure #3, 30 min following exposure #3, 5 h following exposure #3, 7.5 days following exposure #3, and 12 days following exposure #3.

Data analysis. Statistical analysis was done by ANOVA with a Bonfer-roni/Dunn posttest. A p value of < 0.05 was used as the criterion for statistical significance. All numerical values are reported as mean ± SEM.

RESULTS

The average sarin vapor concentration generated was 4.13 ± 0.07 mg/m³. Other than miosis, the rats did not show any overt clinical signs of nerve agent exposure (i.e., tremors, salivation, rhinorrhea) and no deaths occurred, confirming that the concentration generated was between the EC_{50} for miosis and the LC_{50} for a 60-min exposure (data not shown).

Ocular Measurements

Figure 1 shows the changes in pupil size following each of the three exposures to sarin vapor. The first exposure to sarin vapor decreased the ratio of pupil radius to iris radius from a preexposure baseline size of 65.7 ± 2.1 to 1.7 ± 0.5 (2.6 ± 0.7% of the preexposure baseline). Prior to the second exposure (24 h later), the pupils of exposed rats were significantly smaller than the baseline pupil size before the first exposure, having only recovered to 52.0 ± 2.6% of the baseline. Immediately after the second exposure, the ratio of pupil radius to iris radius was decreased to 6.5 ± 2.0, or 18.0 ± 4.6% of the baseline pupil size before the second exposure. Prior to the third exposure, the pupils of exposed rats were significantly smaller than the baseline pupil size before the first exposure, having recovered to 76.4 ± 6.1% of the baseline pupil size before the first exposure. Immediately after the third exposure, the ratio of pupil radius to iris radius was decreased to 36.7 ± 6.0, or 73.3 ± 7.8% of the baseline pupil size before the third exposure.

The pupillary light reflex was used to assess parasympathetic function in the eye at several time points pre- and post-exposure. Figure 2 shows the effect of multiple exposures to sarin vapor on the pupillary light reflex. Twenty-four hours following the third exposure, the pupillary light reflex was greatly attenuated (Fig. 2). The reflex slowly recovered to normal, and was of normal magnitude and duration by approxi-mately 96-h postexposure (data not shown).

Cardiac Measurements

Table 1 shows the effect of sarin exposure on AChE and BChE activities in the blood. Exposure to sarin vapor resulted in a significant decrease in the activity of AChE in the blood relative to baseline values (202 ± 40 U/l postexposure vs. 1213 ± 59 U/l at baseline). AChE activity recovered to 420 ± 94 U/l at 24 h following the first exposure, but was decreased to
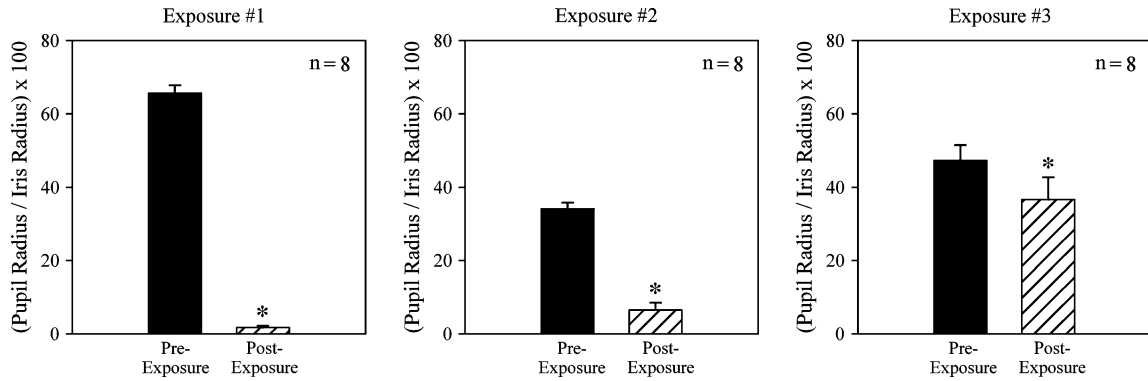
FIG. 1. Pupil size following multiple exposures to sarin vapor. Pupil size, expressed as (pupil radius/iris radius) $\times$ 100, was decreased by greater than 90% at 15 min following the first exposure to sarin vapor (left graph). Fifteen minutes following the second exposure, pupil size was again decreased, although not to the extent that it was following exposure #1 (center graph). Fifteen minutes following the third exposure (right graph), pupil size was again significantly decreased, although the magnitude of the change was significantly less than the response seen following the other two exposures, demonstrating the development of tolerance to the miotic effect of sarin vapor; * denotes $p < 0.05$ when compared to the pre-exposure baseline.

144 ± 18 U/l following the second exposure to sarin vapor. Immediately before the third exposure AChE activity had recovered to 361 ± 26 U/l. Thirty minutes following the third exposure to sarin vapor AChE activity was 192 ± 13 U/l. The recovery of AChE activity following the third exposure was gradual over the next 12 days, when it reached approximately 80% of the baseline value. BChE activity was not significantly changed following the first exposure (395 ± 26 U/l postexposure vs. 346 ± 9 U/l preexposure), the second exposure (295 ± 9 U/l postexposure vs. 318 ± 5 U/l preexposure), or the third exposure (370 ± 17 U/l postexposure vs. 383 ± 18 U/l preexposure).

Parasympathetic tone in the heart was assessed by measuring the change in heart rate induced by injection of atropine (5 mg/kg im; Fig. 3). In unexposed animals, atropine produced an

average increase in heart rate of 104 ± 3 bpm. At the same time point when the pupillary light reflex was greatly attenuated (24 h following the third exposure), atropine produced an average increase in heart rate of 121 ± 11 bpm. Four days following the third exposure, atropine produced an average increase in heart rate of 100 ± 11 bpm. Fourteen days following the third exposure, atropine produced an average increase in heart rate of 122 ± 15 bpm. There was not a statistical difference between the changes in heart rate induced by atropine at any of the time points investigated.

Changes in heart rate and time domain indices of HRV were assessed following administration of atropine, propranolol, and saline. These changes are summarized in Table 2. In unexposed animals, administration of atropine produced a significant decrease in both SDNN and rMSSD, and a significant increase
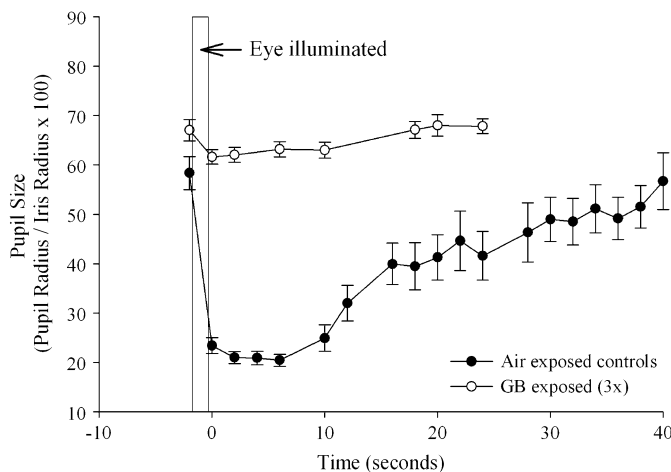

FIG. 2. Effect of multiple exposures to sarin vapor on the pupillary light reflex. In air-exposed animals (black circles), pupil size decreased following illumination of the eye for 2 s, and slowly recovered to the baseline size over the course of the next 60 s. However, in animals exposed to sarin vapor three times (white circles), the pupillary response to light was absent, an indication of parasympathetic dysfunction in the eye; n = 6–8 for each group.

TABLE 1
Effect of Exposure to Sarin Vapor on Whole-Blood Cholinesterase Activity

Time	Whole-blood AChE (U/l)	Whole-blood BChE (U/l)	n
Preexposure	1213 ± 59	346 ± 9	4
30-min postexposure 1	202 ± 40*	395 ± 26	4
60-min preexposure 2	420 ± 94*	318 ± 5	4
30-min postexposure 2	144 ± 18*	295 ± 9	4
60-min preexposure 3	361 ± 26*	383 ± 18	4
30-min postexposure 3	192 ± 13*	370 ± 17	4
5-h postexposure 3	245 ± 12*	374 ± 14	4
7.5-days postexposure 3	804 ± 18*	372 ± 10	4
12-days postexposure 3	955 ± 52*	420 ± 6	4

Note. Whole-blood AChE activity was significantly inhibited relative to the baseline value throughout the exposure sequence. Following the third exposure, whole-blood AChE activity slowly returned toward the baseline value. Whole-blood BChE activity was not significantly decreased following any of the exposures when compared to the appropriate preexposure value; * denotes value is significantly less ($p < 0.05$) than the baseline value.
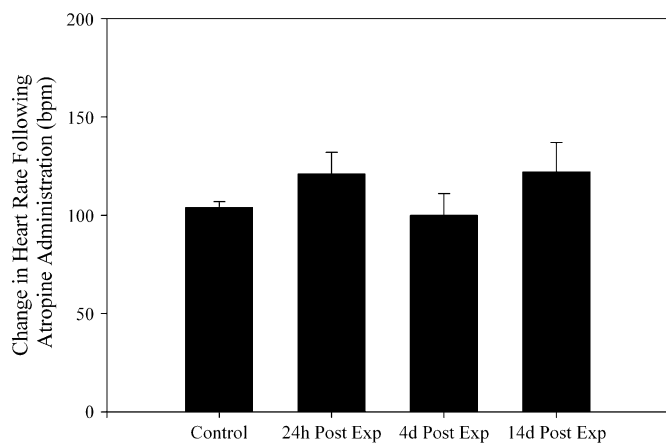
FIG. 3. Effect of atropine on heart rate following multiple exposures to sarin vapor. In unexposed animals, atropine produced an average increase in heart rate of 104 ± 3 bpm. In animals exposed to sarin vapor for 3 consecutive days, the change in heart rate induced by atropine administration at 24-h, and 4- and 12-days postexposure was not significantly different from that in control animals; $n = 5$ for each bar.

TABLE 2
Effects of Atropine and Propranolol on Time Domain Indices of HRV

Time point	Heart rate (bpm)	SDNN (ms)	rMSSD (ms)
Post-saline	364 ± 8	4.27 ± 0.27	2.07 ± 0.13
Post-atropine	$463 \pm 10*$	$1.76 \pm 0.11*$	$0.73 \pm 0.44*$
Post-saline	322 ± 8	5.37 ± 0.26	2.47 ± 0.12
Post-propranolol	$300 \pm 5*$	$3.56 \pm 0.40*$	2.28 ± 0.25

Note. Administration of atropine (5 mg/kg im) resulted in a significantly increased heart rate and significantly decreased SDNN and rMSSD values relative to the injection of saline the day before. Administration of propranolol (5 mg/kg im) resulted in a significantly lower heart rate, and a significantly decreased SDNN relative to the injection of saline the day before. Heart rate, SDNN, and rMSSD values are the average for the first 20 min following administration of the treatment; $n = 5$ for each treatment; * denotes $p < 0.05$ when compared to saline treated animals.

in heart rate, when compared to saline injected controls (Table 2). Administration of propranolol produced a significant decrease in SDNN and heart rate, but no change in rMSSD, when compared to saline injected controls (Table 2). Administration of saline produced a small increase in heart rate, but did not produce a significant change in either SDNN or rMSSD relative to the preinjection baseline (data not shown). Based on these results and the results of previous studies, SDNN can be used as an indicator of total autonomic input to the heart, whereas rMSSD can be used as an indicator of parasympathetic input to the heart (El-Mas and Abdel-Rahman, 2003; Sgoifo et al., 1997; Stein et al., 1994). In animals exposed to sarin vapor, neither the SDNN nor the rMSSD was significantly decreased at 2 h, 1, 4, 7, or 14 days following exposure #3 relative to the preexposure baseline (Figs. 4A and 4B). Additionally, the heart rate in animals exposed to sarin vapor was not significantly changed at 2 h, 1, 4, 7, or 14 days following exposure #3 relative to the preexposure baseline (Fig. 4C). Air-exposed animals did not show any changes in SDNN, rMSSD, or heart rate at 1-day or 7-days postexposure (data not shown).

DISCUSSION

The dose of sarin used was sufficient to produce pinpoint pupils in exposed animals, a result of overstimulation of the parasympathetic branch of the autonomic nervous system in the eye. This response was expected based upon dose–response data reported previously for sarin vapor in the rat (Mioduszewski, 2002). Recovery from sarin-induced miosis was complete by 24 h following the third exposure. Once recovered, the pupil exhibited an attenuated response to light, suggesting the presence of dysfunction of the parasympathetic nervous system. Additionally, the magnitude of the miotic response to sarin

vapor was decreased following the third exposure relative to the first and second exposures, again suggesting the presence of parasympathetic dysfunction. It is well known that muscarinic receptors desensitize following prolonged stimulation, and this desensitization has been reported in several tissues, including the heart (Shui et al., 2002), eyes (Soli et al., 1980), and the gastrointestinal tract (Mita et al., 1997). These results support the findings from studies demonstrating parasympathetic dysfunction due to desensitization of muscarinic receptors following prolonged cholinergic stimulation (Bito and Banks, 1969; Dabisch et al. 2005a,b; Soli et al., 1980).

Given the similarities between the autonomic innervation in the eye and the heart, it is possible that parasympathetic dysfunction could also occur in the heart following exposure to an inhibitor of AChE. Therefore, the present study assessed HRV in conscious rats to investigate the hypothesis that multiple exposures to the nerve agent sarin would result in muscarinic receptor desensitization and parasympathetic dysfunction in the heart. HRV is a useful tool for the evaluation of cardiac autonomic tone. A decrease in HRV has been shown to have prognostic value for acute myocardial infarction, diabetic autonomic dysfunction, and sudden cardiac death (Singer et al., 1988; Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology, 1996). Additionally, HRV is useful in the evaluation of drugs and chemicals that may influence cardiac autonomic balance (Pentilla et al., 2005). In the present study, administration of atropine resulted in a significant decrease in both measures of HRV examined, suggesting the presence of significant parasympathetic tone in the heart of unexposed animals. A similar result has been reported previously in humans treated with anticholinergic drugs (Pentilla et al., 2005). Administration of propranolol resulted in a significant decrease in the SDNN, but not the rMSSD. These results are in agreement with several studies that suggest that SDNN reflects both sympathetic and
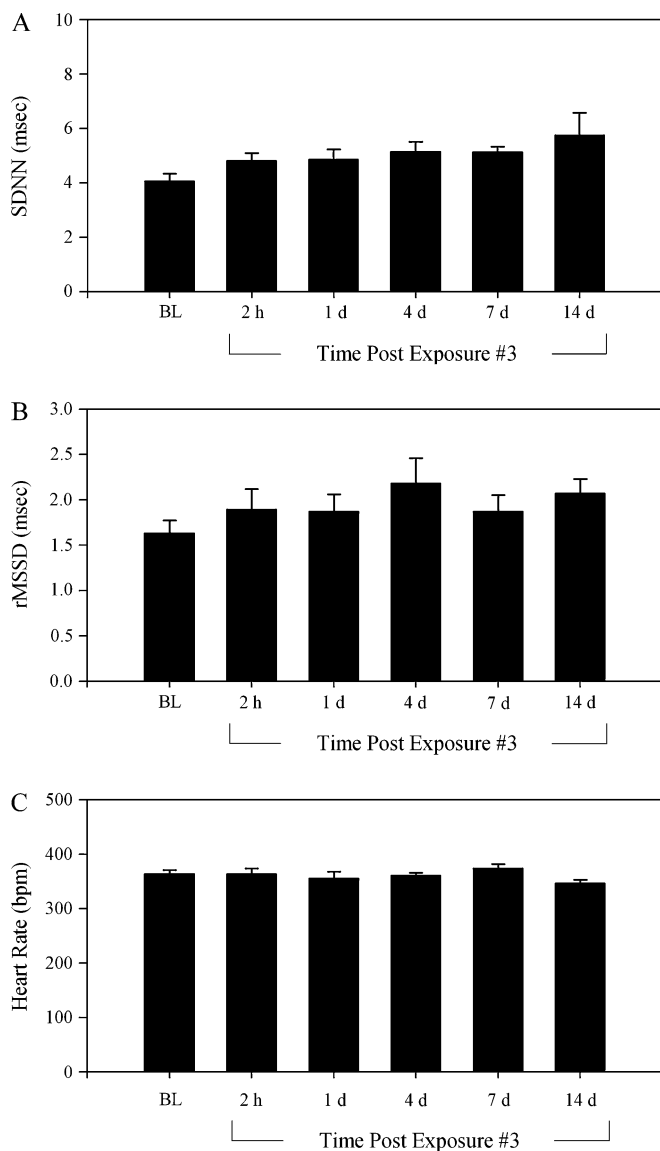
FIG. 4. Effect of multiple exposures to sarin vapor on time domain indices of HRV. (A) The SDNN was not significantly different from the preexposure baseline (BL) at 2-h, 1-, 4-, 7-, or 14-days postexposure; (B) the rMSSD was not significantly different from the preexposure baseline (BL) at 2-h, 1-, 4-, 7-, or 14-days postexposure; (C) the heart rate was not significantly different from the preexposure BL at 2-h, 1-, 4-, 7-, or 14-days postexposure; $n = 6$ for each bar.

parasympathetic input to the heart, whereas rMSSD reflects only the parasympathetic input to the heart (El-Mas and Abdel-Rahman, 2003; Sgoifo et al., 1997; Stein et al., 1994).

It was hypothesized that multiple exposures to sarin vapor would result in parasympathetic dysfunction involving desensitization of cardiac muscarinic receptors, similar to what has been reported both in isolated rat atrial cells (Shui et al., 2002) and in the eye of the rat, monkey, and guinea pig (Bito and Banks, 1969; Dabisch et al., 2005a, b; Soli et al., 1980). If cardiac muscarinic receptors desensitized following multiple

exposures to sarin vapor, it would be expected that HRV, in particular the rMSSD, would be decreased in a manner similar to that seen following atropine administration. However, the rMSSD was not significantly different from baseline at any time point investigated postexposure, suggesting that desensitization of cardiac muscarinic receptors did not occur. Additionally, the SDNN was not significantly different from baseline at any time point investigated postexposure, suggesting that total autonomic input to the heart was not altered following the exposures. Blockade of muscarinic receptors in the heart with atropine produced an increase in heart rate in animals exposed to sarin vapor that was not significantly different than that produced in unexposed animals, again suggesting that the parasympathetic nervous system and cardiac muscarinic receptors were functional.

Many studies have examined the effects of cholinesterase inhibitors, including nerve agents, on the heart. Several studies have reported that QT interval prolongation in rats following exposure to sarin or soman for several weeks postexposure (Abraham et al., 2001; Allon et al., 2005). Additionally, Allon et al. (2005) demonstrated a decreased threshold for the induction of arrhythmias in rats exposed to near lethal levels of sarin for several months postexposure. Lesions in cardiac tissue have also been reported following exposure to near lethal doses of sarin (Abraham et al., 2001; Singer et al., 1987). In contrast to the results of the present study, several studies have also found alterations in HRV following exposure to cholinesterase inhibitors. Soares et al. (2004) reported that pyridostigmine increased time domain indices of HRV, namely SDNN, in rats following administration in drinking water for 7 days. Similarly, a study by Morris et al. (2007) reported that mice injected with a $0.05 \times LD_{50}$ of sarin showed an increase in HRV. This initial increase in HRV was followed by a significant decrease in HRV at 2 months postexposure (Morris et al., 2007). In the present study, the average concentration of sarin for each of the three exposures was 4.13 ± 0.07 mg/m^3 for 60 min, a dose approximately equivalent to $0.5 \times LC_{50}$ for sarin in male rats at this exposure duration (Miodusweski et al., 2002). However, it is not clear how the inhaled dose used in the present study relates to oral or injected nerve agent. It may be possible to compare the doses based on the degree of inhibition of AChE in the blood found in these other studies since, regardless of the route of administration, the agent must enter the bloodstream first in order to reach the heart. In the study by Soares et al. (2004), pyridostigmine administration produced an average decrease in blood AChE activity of 40%. Surprisingly, in the study by Morris et al. (2007), administration of sarin did not produce a significant change in blood AChE activity. In the present study, exposure to sarin vapor produced a decrease in whole-blood AChE activity of approximately 80%. Thus, unexpectedly, the degree of AChE depression in the blood does not appear to correlate with the effect on HRV. There are no obvious differences between these studies that could account for the observed differences. Therefore, the

reason for this discrepancy remains unclear. Additional studies utilizing several different doses of sarin may help to clarify this issue.

In the rat, different muscarinic receptor subtypes mediate responses to cholinergic stimulation in the eye and in the heart. In the eye, the M_3 subtype is the predominant form present, while in the heart the M_2 subtype is the predominant form present (Krejci and Tucek, 2002; Shiraishi and Takayanagi, 1993). Several studies have demonstrated desensitization of atrial muscarinic receptors (Hosey et al., 1995; Shui et al., 2002) or decreased cardiac muscarinic receptor numbers (Myslivecek et al., 1996; Vallette et al., 1997) in rats exposed to cholinesterase inhibitors. Similarly, muscarinic receptors mediating the miotic response have been shown to desensitize following excessive stimulation (Bito and Banks, 1969; Soli et al., 1980). These studies demonstrate that both ocular and cardiac muscarinic receptors are susceptible to changes in function due to overstimulation. Thus, it is unlikely that the observed difference in the response in the eyes and the heart is because the M_3 muscarinic receptors present in the heart are not able to be desensitized.

In the present study, whole-blood AChE activity was depressed by greater than 80% relative to the baseline level following each exposure to sarin vapor, demonstrating that sarin was reaching the bloodstream and presumably distributing throughout the body. However, based on the lack of effect on autonomic tone in the heart, it appears that the amount of sarin in the bloodstream was not sufficient to inhibit cardiac AChE. Additional studies incorporating the measurement of cardiac AChE activity would be helpful in determining whether or not sarin was present in sufficient amounts to inhibit AChE in the heart; however, this was not possible in the present study since the animals were followed for several weeks post-exposure.

The lack of parasympathetic dysfunction in the heart is in contrast to the dysfunction that develops in the eye at the same dose of sarin. This is likely a function of the route of sarin exposure. It has been demonstrated previously that covering the eyes during a nerve agent vapor exposure is able to prevent the development of miosis (Sim, 1956). Thus, miosis induced by nerve agent vapor is a result of nerve agent penetration directly into the eye, and not absorption through the lungs and subsequent redistribution to the eyes via the blood. Thus, it is possible that more nerve agent is reaching the eye than is reaching the heart since agent penetrating the eye is not bound or metabolized in the blood or other tissues before it reaches the pupillary sphincter muscle.

In summary, rats exposed on multiple occasions to sarin vapor developed parasympathetic dysfunction in the eye characterized by an attenuated response to light and a diminished miotic response to sarin vapor exposure. However, the same dose of sarin vapor failed to produce any effects on either time domain indices of HRV or the magnitude of the tachycardia induced by atropine, suggesting that autonomic control in the heart was not affected. It is possible that the dose of sarin used in the present study was insufficient to inhibit cardiac AChE, explaining the lack of a response in the heart. Additional studies which utilize higher doses of sarin may be able to inhibit cardiac AChE, resulting in overstimulation of cardiac muscarinic receptors, ultimately resulting in desensitization and parasympathetic dysfunction.

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