Epinephrine-induced arrhythmias (EPIA) are known to be associated with local cardiac cholinergic activation. The present study examined the development of QT prolongation and the effect on EPIA of whole-body exposure of animals to a potent acetylcholine esterase inhibitor. Freely moving rats were exposed to sarin vapor (34.2 ± 0.8 μg/liter) for 10 min. The electrocardiograms (ECG) of exposed and control animals were monitored every 2 weeks for 6 months. One and six months post exposure, rats were challenged with epinephrine under anesthesia, and the threshold for arrhythmias was determined. Approximately 35% of the intoxicated rats died within 24 h of sarin exposure. Additional occasional deaths were recorded for up to 6 months (final mortality rate of 48%). Surviving rats showed, agitation, aggression, and weight loss compared to non-exposed rats, and about 20% of them experienced sporadic convulsions. Sarin-challenged rats with severe symptoms demonstrated QT segment prolongation during the first 2–3 weeks after exposure. The EPIA that appeared at a significantly lower blood pressure in the treated group in the first 2 weeks for 6 months. One and six months post exposure, rats were challenged with epinephrine under anesthesia, and the threshold for arrhythmias was determined. Approximately 35% of the intoxicated rats died within 24 h of sarin exposure. Additional occasional deaths were recorded for up to 6 months (final mortality rate of 48%). Surviving rats showed, agitation, aggression, and weight loss compared to non-exposed rats, and about 20% of them experienced sporadic convulsions. Sarin-challenged rats with severe symptoms demonstrated QT segment prolongation during the first 2–3 weeks after exposure. The EPIA that appeared at a significantly lower blood pressure in the treated group in the first month after intoxication lasted for up to 6 months. This decrease in EPIA threshold was blocked by atropine and methyl-atropine. Month after intoxication lasted for up to 6 months. This decrease in EPIA threshold was blocked by atropine and methyl-atropine.

Key Words: rat; sarin; inhalation toxicity; QT prolongation; epinephrine-induced arrhythmias.

INTRODUCTION

The increase in accidental organophosphorous poisoning as well as the rise in the number of cases of suicide attempts with organophosphates (OPs) is due primarily to the widespread use of these compounds in agriculture. Moreover, in recent years, OPs have also been used in acts of terror (Okumura et al., 2003). Once they enter the central nervous system (CNS), OPs are powerful inhibitors of cholinesterases (ChEs). As a consequence, they cause a massive accumulation of acetylcholine (ACh) and overstimulation of cholinergic synapses of central and peripheral nerve endings. The cardiac effect of OP poisoning is characterized by the profound upsurge in sympathetic tone expressed as sinus tachycardia, and the robust increase in parasympathetic tone displayed by S-T segment changes, abnormal atrio-ventricular (A-V) conduction, and arrhythmias. Notably, QT interval prolongation and sudden death are also distinct features of cardiac OP intoxication (Kiss and Fazekas, 1979; Roth et al., 1993).

Life-threatening delayed cardiotoxicity resulting from exposure to OP compounds was first reported by Luzhnikov et al. (1975). Their study reported on cardiac follow-up of 183 cases of severe OP intoxication admitted to the hospital during 1972. About 18% of the patients died from various arrhythmias and conduction disturbances. Patients with arrhythmias had a prolonged QT interval that was correlated with the severity of intoxication. In these patients, QT prolongation developed into polymorphous arrhythmias and delayed death (Ludomirsky et al., 1982; Wang et al., 1998). However, QT prolongation could not account for all cases of heart failure resulting from OP exposure, because patients without this effect also suffered from arrhythmias and cardiac and respiratory failure (Chuang et al., 1996). Therefore, other factors need to be identified to fully explain this delayed effect of OP poisoning.

Abnormalities in ECG patterns and arrhythmias that developed after OP administration were also observed in experimental animals, such as dogs (Das et al., 1985; Dayrit et al., 1948) and rats (McDonough et al., 1995; Singer et al., 1987). QT prolongation was recorded in rats for up to 3 months after exposure to acute OP administration (Abraham et al., 2001). These rats also demonstrated increased sensitization to epinephrine: EPIA developed at lower doses in the OP-exposed rats than in control animals (Abraham et al., 2002). In the present study we tested the long-term effects of inhalation exposure of sarin in freely moving rats. We used the epinephrine challenge method to detect alterations in cardiac function. In addition, to elucidate the underlying mechanism.
for the development of arrhythmias and delayed death induced by OP intoxication, we looked for cholinergic manipulations after epinephrine challenge.

MATERIALS AND METHODS

Materials. 3H-N-methyl scopolamine (specific activity 83.5 Ci/mmol), was purchased from PerkinElmer (Boston, MA). Epinephrine, atropine sulfate, atropine methyl nitrate (methyl-atropine), physostigmine, pyridostigmine bromide, and carbamylcholine chloride were purchased from Sigma-Aldrich (Ness Ziona, Israel).

Animals. Male albino Sprague-Dawley rats weighing 300–350 g at the beginning of the experiment were purchased from Charles River (England). Care and maintenance were in accordance with the principles described in the Guide for Care and Use of Laboratory Animals (NIH Publication 85–23, 1985). Animals were housed in mesh cages, three per cage, in a controlled environment with a constant temperature of 21° ± 2°C and a 12-h light/dark cycle. Food and water were available ad libitum.

Inhalation exposure. Awake, freely moving rats were exposed to sarin vapor in an inhalation apparatus described previously (Allon et al., 1998; Biton and Aharonson, 1978), and modified for whole-body exposure. The exposure system was placed in a hood that operated under sub-atmospheric pressure (~20 cm water). The exposure system was an assembly of two separate lines of continuous flow of clean air and sarin-contaminated air at the required concentration. Sarin vapor was introduced into the exposure chamber by pneumatic valves for short and controlled administration of the OP.

Twenty to 30 min before the exposure, rats were placed for adaptation in a stainless-steel grid cage. The cage was then placed in the exposure chamber for 10 min of adaptation to the flow of clean air. Following the determination of LC50 (see below), groups of 6 rats were exposed to 34.2 ± 0.8 g/l of sarin vapor for 10 min in an exposure chamber designed for fast (1 min) build-up and an even distribution using a computational fluid dynamics (CFD) simulation program (Phoenics CFD code, Technology Services, The University of Iowa, USA). At the end of the exposure period, clean air was reintroduced into the chamber.

The control group was processed as above, except that the animals were exposed to non-contaminated clean air only. Toxicity signs were recorded throughout exposure and for 24 h thereafter.

Sarin analysis. The concentration of sarin in the inhaled air was monitored on-line using a Fourier transform infrared (FTIR) analyzer (Bruker, Karlsruhe, Germany) equipped with specific gas chamber (Infrared Analysis, Anaheim, CA). The peak values determined from the FTIR measurements were calibrated by gas chromatography/mass spectrometry (GC/MS) analysis using sarin and Sarin oxide as standards.

Inhaled median lethal concentration (LC50). Five groups of 6 rats each were exposed to different concentrations of sarin vapor (31.4–35.0 g/l) for 10 min. The LC50 was calculated using the Spearman-Karber method (Finney, 1964).

Preparation of animals for cardiac monitoring. Rats were anesthetized with Equithesine (chloral hydrate, MgSO4, propylene glycol, Nembutal, and ethanol) 100 g b.w. and the right carotid artery and the right femoral artery were cannulated for intra-carotid injection and monitoring of arterial blood pressure (BP), respectively. Arterial blood pressure and lead II of the ECG were recorded (Gould recorder model RS-3400 [USA/UK] or Biopack System model MP-100, [Goleta, CA]). Arrhythmias were identified by the generation of preventricular beats (PVBs) simultaneously with a decrease in the blood pressure.

Epinephrine induced arrhythmias. The intra-carotid cannula was placed with the tip close to the heart ostium. Therefore, each injection affected the heart directly regardless of the animals’ weight. Epinephrine (0.25–100 µg/rat) was administered by intra-carotid injection in 0.1 ml of saline, followed by an 0.2 ml saline wash to remove the residual drug. Each animal received increasing doses every 5 min until arrhythmias were induced by two consecutive injections.

Pyridostigmine or physostigmine (0.1 mg/kg each) were administered to the rats by intra-carotid injection 10 min after the threshold for EPIA was determined and heart rate and blood pressure had returned to baseline. The new threshold was determined 5 min post drug injection. A similar experimental paradigm was used for atropine or methyl-atropine (1 mg/kg each).

ECG. Lightly anesthetized rats (Halothane, Zeneca, UK) were used for ECG monitoring, recorded every 2 weeks for up to 6 months post exposure, and QT intervals were measured. QT intervals were normalized to the heart rate according to the following equation:

QTc = QT/√R-R interval,

where QTc is the corrected value of QT and R-R is the time interval between two consecutive heart beats.

Binding experiments. Five months post exposure, rats were decapitated and their hearts and brains were rapidly removed and immediately frozen in liquid N2 and kept at −80°C until used. Tissues were homogenized in 15 volumes of 20 mM Tris-HCl buffer (containing 320 mM sucrose, pH 7.4). Homogenates were centrifuged at 10,000 x g for 10 min at 4°C. Supernatants were centrifuged at 40,000 x g for 20 min at 4°C, and the pellets were resuspended in the assay buffer (50 mM phosphate buffer containing 2 mM MgCl2, pH 7.4). Binding parameters of 3H-N-methyl scopolamine (3H-NMS) were determined by incubating six concentrations of this ligand with membrane preparations for 60 min at 25°C in a final volume of 1 ml. Bound 3H-NMS was separated from free ligand by rapid filtration, and nonspecific binding was determined in the presence of 10 µM atropine.

For carbachol competition curves, various concentrations of this agent were used to displace 3H-NMS bound to heart membranes. Protein concentrations were determined with the Bradford method (Bradford, 1976).

Data and statistical analyses. Binding parameters were assessed by computer-assisted non-linear least squares regression analysis, with the Prism4 program (GraphPad Software, Inc.). Data are presented as means ± SEM, and statistical difference between groups was assessed by analysis of variance (ANOVA). A value of p < 0.05 was accepted as statistically significant.

RESULTS

The LC50 for 10 min of sarin inhalation was 342.6 µg × min/l (95% confidence limits: 335–350). Surviving rats were divided into three groups: (1) sarin-exposed with moderate to severe symptoms characterized by long-lasting tonic-clonic convulsions; (2) sarin-exposed with no convulsions and almost no visible signs; (3) control group exposed to clear air. Approximately 35% of the sarin-exposed rats died within the first 24 h. An additional 10% died during the first week, and sporadic deaths at the rate of approximately 5% per month were recorded during the 6-month follow-up period. It should be noted that none of the control rats died during the observation period.

Rats showing moderate to severe symptoms at the time of exposure, but not those with virtual absence of symptoms,
showed a significant decrease in body weight (10–18%) compared to control animals. Two-way ANOVA with repeated measures (groups × weeks) demonstrated an interaction \( F(12,126) = 2.3, p = 0.011 \), and Dunnett’s post-hoc analyses for week 2 up to week 13 (4 months post exposure) yielded significant differences between the severe symptoms and control groups \( (p = 0.03) \). Although the QTc of rats from groups 2 and 3 were within the normal range (Osborne, 1981), the QTc of the first group with severe symptoms was significantly higher 2 weeks post exposure (Fig. 1). However, these values returned to the control level within the first 4 weeks after treatment. Notably, 4 (out of 10, filled triangles) rats from this group (Fig. 1, insert), showed pronounced QTc prolongation starting 13 weeks post exposure and this feature remained high for the rest of the follow-up period; in one case, QTc prolongation developed into spontaneous arrhythmias 5 months post exposure.

At both 1 month and 6 months after exposure, rats were challenged by intra-carotid injection of epinephrine. Epinephrine caused positive ionotropic and chronotropic effects on the heart, concomitant with an increase in blood pressure (BP). Recovery from high BP started roughly 2 min post injection, the pressure was reduced below normal at about 4 min, and it returned to normal approximately 5 min after epinephrine injection.
Arrhythmias Caused by Epinephrine in Sarin-Exposed Rats

<table>
<thead>
<tr>
<th>Threshold of EPIA</th>
<th>Number of PVBs</th>
<th>Onset (s)</th>
<th>Delta BP (mm Hg)</th>
<th>BP (mm Hg)</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.4 ± 1.3</td>
<td>34.1 ± 6.0</td>
<td>9.0 ± 0.6</td>
<td>53.1 ± 6.5</td>
<td>130.4 ± 10.2</td>
<td>Control (naive) (7)</td>
</tr>
<tr>
<td>Sarin exposed</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.9 ± 0.7*</td>
<td>16.0 ± 3.9*</td>
<td>15.8 ± 2.5</td>
<td>49.0 ± 6.8</td>
<td>107.0 ± 6.7</td>
<td>No treatment (13)</td>
</tr>
<tr>
<td>4.1 ± 1.6</td>
<td>34.8 ± 16.3</td>
<td>13.8 ± 2.7</td>
<td>51.4 ± 12.9</td>
<td>98.8 ± 11.2</td>
<td>Pyridostigmine (5)</td>
</tr>
<tr>
<td>4.5 ± 2.8</td>
<td>17.3 ± 12.8</td>
<td>18.7 ± 9.3</td>
<td>45.3 ± 8.6</td>
<td>103.7 ± 10.7</td>
<td>Physostigmine (3)</td>
</tr>
<tr>
<td>12.5 ± 5.1*</td>
<td>2.4 ± 0.7*</td>
<td>26.4 ± 10.1</td>
<td>67.0 ± 15.2</td>
<td>119.2 ± 12.2</td>
<td>Methyl atropine (5)</td>
</tr>
<tr>
<td>21.7 ± 14.5**</td>
<td>2.7 ± 0.9</td>
<td>20.0 ± 1.8</td>
<td>84.0 ± 7.3**</td>
<td>131.3 ± 16.1</td>
<td>Atropine (3)</td>
</tr>
</tbody>
</table>

Note. Intra-carotid injection of epinephrine induces arrhythmias characterized by various parameters at onset (e.g., BP, number of premature ventricular beats [PVBs], etc.). Epinephrine was injected at various doses until arrhythmias were noted. Pyridostigmine and physostigmine (0.1 mg/kg) or atropine and methylatropine (1 mg/kg) were injected 5 min before challenge with epinephrine. The number of animals per group is given in parentheses. Asterisks indicate significant difference from the control group. *p < 0.05; **p < 0.01. EPIA: epinephrine-induced arrhythmias.

The Effect of Sarin Vapor on 3H-NMS Binding Characteristics in Various Tissues of the Rat

<table>
<thead>
<tr>
<th>Treatment Tissue</th>
<th>Control (n = 4)</th>
<th>Sarin (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>kᵦ (pM)</td>
<td>kᵦ (pM)</td>
</tr>
<tr>
<td>Heart</td>
<td>269.8 ± 36.3</td>
<td>237.7 ± 11.0</td>
</tr>
<tr>
<td></td>
<td>135.7 ± 9.4</td>
<td>137.4 ± 15.4</td>
</tr>
<tr>
<td>Hippocampus</td>
<td></td>
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</tbody>
</table>

Note. Binding experiments were performed as described in Materials and Methods. Muscarinic receptor densities (Bₘₐₓ) and dissociation constant (kᵦ) are presented as means ± SEM of four independent determinations for each group.

DISCUSSION

This report describes a novel system designed for exposure of freely moving animals to sarin vapor. The technique is distinguished by use of an exposure chamber in which the inlet and outlet paths were designed for fast (1 min) build-up and an even distribution of vapor concentration by means of a CFD simulation program. Additionally, online FTIR analysis of the
sarin concentration, calibrated by chemical analysis, was incorporated into the system. This enabled reliable and reproducible repeat exposures throughout the entire experiment.

Data reported herein demonstrate that rats administered 34.2 ± 0.8 μg/l sarin for 10 min undergo long-term impairment of cardiac performance, as noted by EPIA, an established measure of cardiac sensitivity (Evris et al., 1987; Opic and Lubbe, 1979). The decrease in EPIA threshold was correlated with the severity of intoxication, evidenced by its early appearance, i.e., at 1 month post exposure in animals with severe clinical symptoms. In these rats, this effect lasted for at least 6 months, the point at which the experiment was terminated. These findings are in accordance with a previous report by Abraham et al. (2002) that showed sensitization of rats to epinephrine after a bolus injection of sarin or soman.

Our results also illustrate that sarin exposure induced QTc prolongation. The effect was limited in most rats to the first 2 weeks after intoxication and was found only in rats presenting critical toxicity signs during or immediately after poisoning. This observation is consistent with reports concerning human accidental exposures demonstrating QT segment prolongation characterized by short duration (2–3 weeks) (Chuang et al., 1996; Kiss and Fazekas, 1979; Ludomirsky et al., 1982; Roth et al., 1993). However, cardiac and respiratory failure leading to delayed deaths also occurred among OP-exposed subjects not experiencing QT prolongation (Chuang et al., 1996). Our experiments involving sarin vapor administration to freely moving rats documented sporadic deaths throughout the follow-up period (6 months), even after the changes in the QT segment returned to the normal pattern (Fig. 1). Notably, these animals with a severe clinical response were marked by an array of clinical symptoms such as convulsion, agitation, and aggression, as well as a decrease in the threshold below which they developed arrhythmias. The fact that the latter indication appeared at markedly lower BP values and epinephrine doses might be the crucial cause for the delayed deaths. However, Abraham et al. (2001) exhibited QTc prolongation persisting for up to 3 months post sarin or soman injections, a disparity that could be due to the different exposure paradigms.

It should be pointed out that episodes of convulsions or aggressive behavior, as well as intensive activity or stress, might induce the release of epinephrine from the adrenal gland (Opic and Lubbe, 1979; Wurtman, 1966). A surge like this could increase blood pressure to a level sufficient for the development of arrhythmias, especially in subjects exhibiting a low threshold for EPIA. For instance, emotional stress can precipitate severe and reversible left ventricular dysfunction in patients without coronary disease (Van Huysduyven et al., 2004) Thus, it is suggested that the long-lasting nature of this phenomenon observed after exposure to OP compounds (e.g., agricultural pesticides), may increase the vulnerability of patients to stress and intensive exercise and may result in sudden death from heart failure. In support of our suggestion, Khositseth et al. (2005) demonstrated that low doses of epinephrine can cause alterations in the electrocardiographic T-wave pattern associated with poor clinical prognosis in patients with QT prolongation, both features typical of OP poisoning. Furthermore, our results show that an increased sensitivity to epinephrine is evident in animals manifesting no symptoms, thus widening the circle of subjects susceptible to cardiac malfunction and death.

These observations gain added gravity and importance in light of the widespread use of OPs in agriculture, which is associated with growing numbers of accidental exposure, many of which terminate in sudden death. Recent threats of terrorist activity provide still more relevance to our findings, because a large number of patients exhibiting mild symptoms could be expected after the deliberate spread of pesticides or chemical warfare agents, as observed in the two incidents of sarin mass-poisoning in Japan (Okumura et al., 2003). Indeed, few of those victims complained of cardiac arrhythmias or showed a decreased cardiac contraction (Okudera, 2002).

REFERENCES


