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A critical role of acute bronchoconstriction in the mortality associated with high-dose sarin inhalation: Effects of epinephrine and oxygen therapies

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ABSTRACT

Sarin is an organophosphate nerve agent that is among the most lethal chemical toxins known to mankind. Because of its vaporization properties and ease and low cost of production, sarin is the nerve agent with a strong potential for use by terrorists and rouge nations. The primary route of sarin exposure is through inhalation and, depending on the dose, sarin leads to acute respiratory failure and death. The mechanism(s) of sarin-induced respiratory failure is poorly understood. Sarin irreversibly inhibits acetylcholine esterase, leading to excessive synaptic levels of acetylcholine and, we have previously shown that sarin causes marked ventilatory changes including weakened response to hypoxia. We now show that LD_{50} sarin inhalation causes severe bronchoconstriction in rats, leading to airway resistance, increased hypoxia-induced factor-1α, and severe lung epithelium injury. Transferring animals into 60% oxygen chambers after sarin exposure improved the survival from about 50% to 75% at 24 h; however, many animals died within hours after removal from the oxygen chambers. On the other hand, if LD_{50} sarin-exposed animals were administered the bronchodilator epinephrine, >90% of the animals survived. Moreover, while both epinephrine and oxygen treatments moderated cardiorespiratory parameters, the proinflammatory cytokine surge, and elevated expression of hypoxia-induced factor-1α, only epinephrine consistently reduced the sarin-induced bronchoconstriction. These data suggest that severe bronchoconstriction is a critical factor in the mortality induced by LD_{50} sarin inhalation, and epinephrine may limit the ventilatory, inflammatory, and lethal effects of sarin.

Introduction

The nerve agents are organophosphates that irreversibly inhibit acetylcholinesterase (AChE), causing the accumulation of acetylcholine and consequent cholinergic toxicity that includes salivation, sweating, lacrimation, meiosis, emesis, seizures, paralysis, respiratory distress, and cardiovascular changes (Leikin et al., 2002). Nerve agents are among the most lethal chemical toxins known to mankind. The four major organophosphorus nerve agents are tabun (GA), sarin (GB, isopropyl methylphosphonofluoridate), soman (GD), and Venom X (VX) (Szinicz, 2005; Wood, 1951). They differ in their volatility, potency, and the ability to cross the blood–brain barrier. Because of its volatility, and ease of production and delivery, sarin is the most likely nerve agent to be used by rouge nations and terrorist groups (Holstege et al., 1997). Sarin exposure causes circulatory, respiratory, and long-and short-term neurological damages (Abou-Donia, 2003; Kadar et al., 1995; Nakajima et al., 1999; Niven and Roop, 2004; Sidell, 1994), and most fatalities in animals and humans result from acute respiratory failure (Sidell, 1994). In the lung, neuronal and various non-neuronal cells produce acetylcholine, and almost all cell types present in the respiratory tract express muscarinic receptors that bind acetylcholine (Racke et al., 2006). Muscarinic receptors control smooth muscle tone and excessive acetylcholine causes bronchoconstriction (Buels and Fryer, 2012). In rats, histopathological analysis has detected acute bronchoconstriction after LD_{50} sarin inhalation, which was resolved within 2–3 weeks in the surviving animals (Pant et al., 1993). We have reported that in rats, inhalation of subclinical doses of sarin induces the expression of proinflammatory cytokines in the brain (Henderson et al., 2002; Kalra et al., 2002; Pena-Philippides et al., 2007); however, inhalation of sarin close to LD_{50} causes ventilatory changes, including weakened responses to hypoxia (Zhuang et al., 2008). In this communication, we present evidence that LD_{50} sarin promotes severe bronchoconstriction, increased levels of the hypoxia-induced factor-1α (HIF-1α), and a proinflammatory cytokine surge that is accompanied with perturbations in lung epithelium and death. Ameliorating bronchoconstriction improves ventilatory responses and cytokine surge, and decreases HIF-1α and mortality in sarin-exposed animals.

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Materials and methods

Animals. Male pathogen-free F344 rats were purchased from Charles River. Animals were housed in class 100 air quality rooms with 12-h light/dark cycle and thermo-neutral temperatures of 25 ± 1 °C. Food (Teklad certified diet) and water were provided ad libitum. Three- to 4-month old animals were used in these experiments. The Lovelace Respiratory Research Institute's (LRRI) Institutional Animal Care and Use Committee approved all animal protocols.

Sarin exposures. Sarin was synthesized and purified by standard procedures and animals were exposed to sarin by inhalation as described previously (Henderson et al., 2002). Briefly, sarin was dissolved in isopropyl alcohol and the animals were exposed to isopropyl alcohol (vehicle control) or sarin by the nose-only exposure system at a concentration of 13–15 mg/m^3 sarin for 10 min (LD_{50}). While some moribund animals were euthanized within 6 h of sarin exposure, generally, control and the sarin surviving animals were euthanized at around 24 h after the exposure.

Oxygen and epinephrine treatments. For 60% oxygen exposure, animals were transferred into specially designed chambers and exposed to 60% oxygen by mixing 100% oxygen with air. Animals were removed from the sarin exposure chamber and placed in chambers with ambient air, 60% O_2, or given epinephrine subcutaneously (12.5 μg/kg body wt) and then placed in ambient air/60% O_2 chambers within 10–20 min after sarin exposure. Animals were monitored and allowed to completely off-gas before handling for subsequent experimental procedures.

Airway resistance. At around 24 h post-sarin-exposure, control and sarin-exposed rats were injected intraperitoneally with pentobarbital (35 mg/kg). If required by response to gentle pinch on the toe, more pentobarbital in 10 mg/kg increments was administered. Once a sufficient plane of anesthesia was reached, a small incision was made in the trachea to insert an 18-gauge needle hub. The cannula was secured by sutures and cyanoacrylate adhesive. The animal was placed on the Flexivent apparatus to measure airway resistance (Singh et al., 2011). At the conclusion of the Flexivent procedure, the animals were euthanized.

Measurement of cardiorespiratory activity and hypoxic responses. Cardiorespiratory activity was carried out after 24 h post-sarin/vehicle exposure on the following groups of animals: (a) 6 control and 9 sarin-exposed, (b) 6 control epinephrine and 9 sarin +epinephrine-treated, and (c) 6 control 60% O_2 and 5 sarin + 60% O_2-treated. The animals were anesthetized with Nembutal (50 mg/kg) injected intraperitoneally. Baseline cardiorespiratory activities and their responses to 10% O_2 for 1 min were measured and analyzed as described previously (Zhuang et al., 2008). After completion of the measurements, the animals were euthanized with intraperitoneal injection of Euthasol (150 mg/kg).

Collection of tissues and bronchoalveolar lavage. Lungs and BAL fluids were collected as described previously (Gundavarapu et al., 2012). Briefly, rats were anesthetized and euthanized by exsanguination, the trachea was surgically exposed and cannulated, and the left lung lobe was tied off with a silk thread suture. The right lobe was lavaged with 20 ml PBS (4×). BAL cells were collected by centrifugation and the supernatant was saved and kept at −80 °C for determination of total proteins and cytokines. The cells (5×10^4) were cytospun on duplicate slides and stained with Diff Quik (Baxter Healthcare, Miami, FL). The slides were read under oil immersion, and at least 200 cells from each slide were counted for cell differential.

Cell and protein contents of BALF. Total protein concentration of lavage fluids was determined by the BCA protein assay kit from Pierce (Rockford, Ill.) with bovine serum albumin as the standard according to the manufacturer's directions.

Histopathology. The left lung was inflated with 10% neutral buffered formalin and immersed in the same solution at a constant pressure for 48 h. The fixed lungs were trimmed in the dorsoventral transverse direction from cranial to caudal to yield serial slices. The tissues were embedded in paraffin, and cut into 5-μm sections. The tissue sections were stained with hematoxylin and eosin, and evaluated by light microscopy for morphological changes.

Quantitative PCR and ELISA assays. For qPCR analysis, total RNA was isolated from the frozen lungs as described elsewhere (Gundavarapu et al., 2012). Briefly, tissues were homogenized in the presence of TRI reagent (MRC Molecular Research Center, Cincinnati, OH), and qPCR was performed with the Step One plus Detection System (Applied Biosystems, Foster City, CA) and the TaqMan One-Step RT-PCR kit containing AmpliTaq Gold DNA polymerase. Specific primers and probes for IL1β, INFγ, TNF-α, IL-2, eotaxin, IL-4, IP-10, HIF-1α, and GAPDH were purchased from ABI (Foster City, CA). One-step RT-PCR was performed with the SuperScript III One-Step RT-PCR System with Platinum Taq. All results were derived from the linear amplification curve and normalized to GAPDH. Fold differences were determined by using the $2^{(-\Delta\Delta CT)}$ method (Livak and Schmittgen, 2001). Cytokines in the BAL fluids were assayed by ELISA as described previously (Singh et al., 2011), using the Cytokine MultiPlex ELISA kit (Biosource-Invitrogen, Camarillo, CA), according to the manufacturer's directions. The sensitivity of the assay for all cytokines was <10 pg/ml.

Metalloproteinase (MMP) analysis. BALF samples (150 μg of protein) were analyzed by gelatin zymograms using 7.5% polyacrylamide gels as described elsewhere (Toth et al., 2012). After electrophoresis, gels were washed in 2.5% Triton X-100 and incubated for 24 h at 37 °C in enzyme buffer (50 μM Tris-HCl, pH 7.5; 5 μM $CaCl_2$; 100 μM NaCl; 1 μM $ZnCl_2$; 0.2 g Brij®-35/L; 2.5 ml of Triton X-100/L; and 0.02 g NaN_3/L). Staining was performed in Coomassie brilliant blue R-250 (2 g/L), and gels were destained by standard procedures. Gelatinolytic bands were measured by densitometry.

Statistical analysis. All data were analyzed with GraphPad Prism software 5.03. One-way ANOVA was used to compare means between the groups by using the Tukey post-hoc test, which compares all groups at 95% CIs. Results are presented as mean ± SD. p values of less than 0.05 were considered statistically significant.

Results

LD_{50} sarin induces severe bronchoconstriction

Sarin enters the human body primarily through inhalation, yet very little is known about the pulmonary toxicity of sarin. The postjunctional muscarinic receptors on smooth muscle and glands are activated by acetylcholine to cause bronchoconstriction and other symptoms of cholinergic toxicity (Mak and Barnes, 1990; Niven and Roop, 2004; Sidell, 1994; Weinbroum, 2004). Rats were exposed to isopropyl alcohol (control) and LD_{50} sarin in groups of 6 and 12 respectively. In the present study, we evaluated the lungs of highly moribund animals by histopathology within 2–6 h and of the surviving animals at 24 h post-sarin exposure. We observed that in sarin-exposed animals, there was perturbation of epithelial mesenchymal interface (Fig. 1A, small arrows) that varied from contractions producing epithelial layer undulations (Fig. 1A, middle panel asterisks) to areas of severely contracted epithelial layer (Fig. 1A, right panel asterisks) leading to partial to total separation of the epithelium in highly moribund animals, shown also by the thickened smooth muscle layer (Fig. 1A, large arrows) and, compared to control, the relative diameter of the alveolar spaces in