

Activation of the Cholinergic Antiinflammatory Pathway Reduces Ricin-Induced Mortality and Organ Failure in Mice

Jon G Mabley,¹ Pal Pacher,² and Csaba Szabo³

¹School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, United Kingdom; ²Section on Oxidative Stress Tissue Injury, Laboratory of Physiological Studies, National Institutes of Health, Bethesda, Maryland, United States of America; ³Department of Anesthesiology, The University of Texas Medical Branch, Galveston, Texas, United States of America

Exposure to ricin, either by accident through ingestion of castor oil plant seeds or intentionally through its use as a bioweapon, invariably leads to multiple organ damage and death. Currently there is only a vaccine in advanced development to ricin, but no other antidote. Ricin causes systemic inflammation with increased proinflammatory cytokine release and subsequent multiple organ failure, particularly kidney and liver dysfunction. Activation of the cholinergic antiinflammatory pathway, specifically through the $\alpha 7$ nicotinic acetylcholine receptor (either indirectly through vagus nerve stimulation or directly through nicotine treatment) reduces proinflammatory gene expression. This activation also increases release of proinflammatory chemokines and cytokines, and has proven effective in a variety of inflammatory diseases. The aim of this study was to investigate whether nicotine treatment protected against ricin toxicity in mice. Male Balb/c mice exposed to ricin had increased serum levels of the inflammatory cytokine tumor necrosis factor- α and markers of both kidney (blood urea nitrogen, creatine) and liver (alanine transaminase) dysfunction, with a subsequent increase in mortality. Nicotine administration 2 h after ricin injection significantly delayed and reduced ricin-induced mortality, an effect coupled with reduced serum levels of tumor necrosis factor- α and markers of kidney and liver dysfunction. Both the kidney and liver had markedly increased cellular oxidative stress following ricin exposure, an effect attenuated by nicotine administration. In conclusion, these data demonstrate that in cases of ricin poisoning, activation of the cholinergic antiinflammatory pathway may prove beneficial by reducing organ damage, delaying mortality, and allowing for a greater chance of survival.

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INTRODUCTION

Ricin is a member of a family of protein toxins whose intracellular target is the 28S rRNA of the 60S ribosomal subunit (1). Ricin causes the depurination of 28S rRNA at a single adenine nucleotide, A4565 in humans and A4256 in mice, which results in inhibition of protein translation. The resulting depurination of 28S initiates the ribotoxic stress response, which is characterized by activation of the stress-activated protein kinases, N-terminal-c-Jun-kinases (JNK), and p38 mitogen-activated protein ki-

nase (MAPK) (2). The resulting activation of these protein kinases modulates the expression of a variety of genes that encode proinflammatory cytokines and chemokines. Ricin has been shown to increase production of proinflammatory cytokines, including tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1), IL-8, and monocyte chemoattractant protein-1 from both primary macrophages and cell lines derived from both mice and humans (3,4) via JNK and p38 activation (3). Similar results have been observed *in vivo*, in cases in which ricin adminis-

tration induced a systemic inflammatory response and multiple organ failure (4–6).

Ricin's wide availability and ease of purification has resulted in its use as a toxic and lethal agent by totalitarian regimes, and more recently, by terrorist groups. In humans, the estimated lethal dose of ricin is 1 to 10 $\mu\text{g}/\text{kg}$ body weight, hence its classification by the Centers of Disease Control and Prevention in the USA as a level-B biothreat (6). There have been more than 750 cases of ricin intoxication, the majority of which have resulted from the ingestion of castor beans, manifested by hemorrhagic diarrhea, liver necrosis, diffuse nephritis, and splenitis (5). Currently there is no antidote or vaccine available against ricin, although significant progress has been made in this area, with prospective vaccines having been tested on human volunteers (7,8). With no antidote available,

Address correspondence and reprint requests to Jon G Mabley, School of Pharmacy and Biomolecular Sciences, University of Brighton, Cockcroft Building, Lewes Road, Brighton BN2 4GJ, UK. Phone: + 44 (0) 1273 642055; Fax: + 44 (0) 1273 679333; Email: j.g.Mabley@brighton.ac.uk.

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the treatment of ricin intoxication consists of removing the toxin from the body as quickly as possible and the use of supportive measures to maintain organ function.

The cholinergic antiinflammatory pathway has been identified as a major neuromodulator of immune-cell function (9,10). Stimulation of the vagus nerve has been demonstrated to protect against endotoxic shock by directly affecting immune cells (11,12). Nicotine has been shown to be antiinflammatory in a variety of animal models of disease states, including colitis (13,14), arthritis (15), and type I diabetes (16), an effect observed in humans (17,18). Nicotine has also proven effective in reducing lung inflammation in asthma, pneumonitis, chronic obstructive pulmonary disease, and acid-induced injury (19,20). The nicotine-mediated reduction in the proinflammatory cytokine profile is common to all disease states against which nicotine has proven protective, including colitis (13,21), arthritis (15), diabetes (16), lung inflammation (19,20), and endotoxic shock (10,12).

With the proinflammatory effects of ricin exposure proposed to be the major effector mechanism of toxicity, the aim of this study was to determine whether nicotine administration protected mice from ricin-induced mortality and organ failure.

MATERIALS AND METHODS

Reagents were obtained from the following sources: ricin was from Vector laboratories (Burlingame, CA, USA); nicotine, thiobarbituric acid, and sodium dodecyl sulfate were from Sigma (St. Louis, MI, USA); BALB/c mice were from Taconic farms (Germantown, NY, USA); and specific cytokine ELISA kits were from R&D systems (Minneapolis, MN, USA).

Animal Studies

In vivo studies were performed in accordance with National Institutes of Health guidelines and with the approval of the local institutional animal care and

use committee. Male BALB/c were injected with ricin (50 or 100 $\mu\text{g}/\text{kg}$ intraperitoneally) \pm either nicotine (0.4 $\mu\text{g}/\text{kg}$, subcutaneously) or vehicle administered at 2 h and 26 h after the ricin injection. The mice were then monitored for mortality over a 48-h period. Control mice and those receiving nicotine alone had the appropriate volumes of saline administered at the required time points throughout the experimental period. Mortality measurements were obtained from two separate experiments, with 10 animals per group with a final *n* of 20 for each treatment protocol. In a second series of studies male BALB/c mice were exposed to ricin (50 $\mu\text{g}/\text{kg}$) \pm either nicotine (0.4 $\mu\text{g}/\text{kg}$, subcutaneously) or vehicle administered 2 h after the ricin injection for 14 h prior to being killed. Serum was then taken for cytokine and biochemical analysis and the liver and kidney were taken for oxidative stress determination; results were obtained from two separate experiments with 5 animals per group, with a final *n* of 10 for each treatment protocol.

Serum TNF- α Levels

The concentration of the proinflammatory cytokine TNF- α was determined in the serum by use of a commercially available ELISA, following the protocol provided by the manufacturer (R&D Systems).

Serum Levels of Alanine Transaminase, Blood Urea Nitrogen, and Creatinine

The serum levels of alanine transaminase (ALT), blood urea nitrogen (BUN), and creatinine were determined enzymatically using an automated Vetscan chemistry analyzer (Abaxis, Union City, CA, USA).

Malondialdehyde Assay

Malondialdehyde (MDA) formation was utilized to quantify the lipid peroxidation in the liver and kidney, measured as thiobarbituric acid-reactive material. Tissues were homogenized (100 mg/mL) in 1.15% KCl buffer; 200 μL of the homogenate was then added to a reaction

mixture consisting of 1.5 mL 0.8% thiobarbituric acid, 200 μL 8.1% sodium dodecyl sulfate, 1.5 mL 20% acetic acid (pH 3.5), and 600 μL distilled H_2O . The mixture was then heated at 90°C for 45 min. After cooling to room temperature, the samples were cleared by centrifugation (10,000g, 10 min), and their absorbance was measured at 532 nm, with 1,1,3,3-tetramethoxypropane as an external standard. The level of lipid peroxides was expressed as nanomoles MDA per milligram protein, which was determined using the Bradford assay (22).

Statistical Analysis

The results are presented as mean \pm SEM; statistical analysis was performed using either one-way analysis of variance followed by Student–Newman–Keuls multiple comparisons *post-hoc* analysis or Kaplan–Meier survival analysis as appropriate, with a *P* value of less than 0.05 considered significant.

RESULTS

Nicotine Treatment Increases Survival following Ricin Administration

Because there is currently no antidote to ricin and the only treatment option is to remove the toxin from the body and provide support to failing organs, any strategy that can extend the survival time to allow for these clinical interventions to become effective would be therapeutically useful. Mice exposed to ricin through a variety of administration routes, including nasal, oral, and intraperitoneal, develop severe inflammatory reactions and organ damage resulting in increased mortality (4,6,23). Nicotine has been identified as antiinflammatory in a variety of disease states, an effect linked to its activation of the cholinergic antiinflammatory pathway through specific nicotinic receptors located on inflammatory cells (24). Activation of the cholinergic antiinflammatory pathway either via nicotine administration or vagal stimulation has proven particularly effective in endotoxic shock induced by injection of lipopolysaccharide,

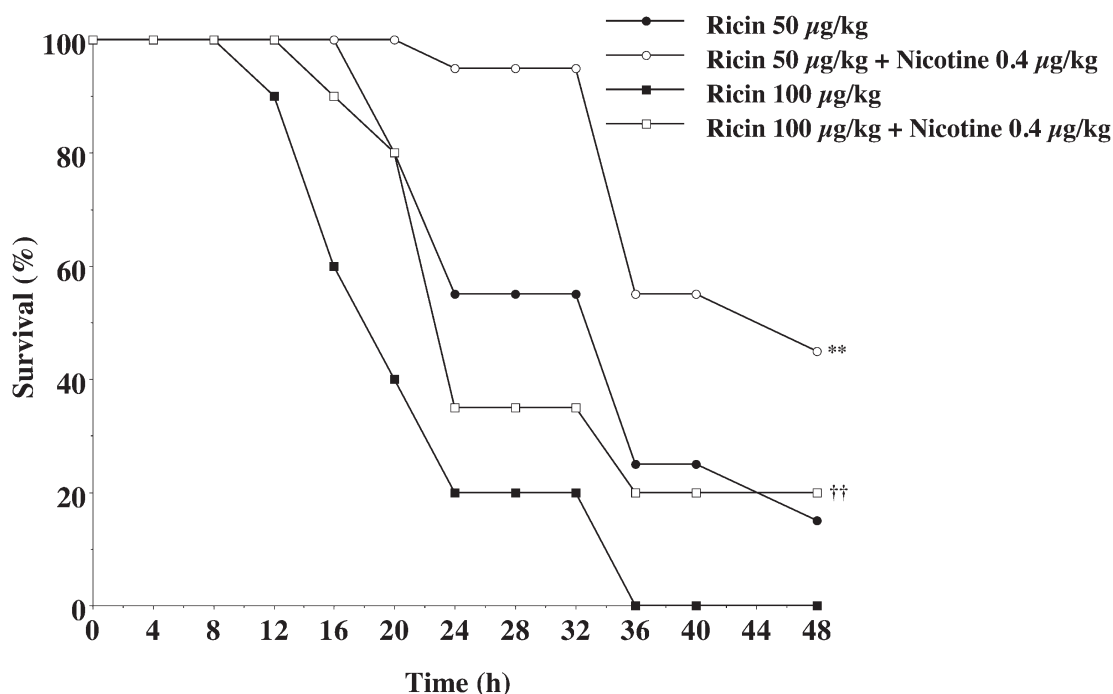


Figure 1. Increased survival of ricin-treated mice following nicotine treatment. Male BALBc mice received an intraperitoneal injection of ricin (50 or 100 µg/kg) followed by a single dose of nicotine (0.4 µg/kg) administered subcutaneously 2 h after the ricin injection. Mouse survival was monitored over a 48-h period. Results are expressed as percentage of surviving mice from 20 animals per group; ***P* < 0.01 versus ricin (50 µg/kg); ††*P* < 0.01 versus ricin (100 µg/kg).

reducing inflammation and, most importantly, improving survival (25). Therefore, we investigated whether nicotine treatment following ricin exposure could delay mortality and increase survival rates. Nicotine treatment was started 2 h after ricin exposure to mimic what is likely to be observed clinically with ricin-exposed patients presenting many hours after the initial contact.

Ricin dose-dependently induced mortality during a 48-h postexposure period, with 50% mortality seen at 24 h with 50 µg/kg and 18 h with 100 µg/kg (Figure 1). Nicotine treatment (0.4 µg/kg) administered at 2 h and 26 h after ricin exposure significantly delayed mortality and improved survival in both the 50 and 100 µg/kg ricin groups (Figure 1), with 45% and 20% of the nicotine-treated mice surviving at 48 h compared with 15% and 0% in the vehicle-treated 50 and 100 µg/kg ricin-treated groups, respectively (Figure 1).

Nicotine Reduces Serum TNF-α Levels following Ricin Exposure

Exposure to ricin has been linked to a marked increase in levels of serum proinflammatory cytokines, including TNF-α, IL-1β, and IL-6 (5). This effect has been proposed to be the major mechanism of ricin-induced organ dysfunction and mortality. Because nicotine delayed mortality and improved survival of mice exposed to ricin, we wanted to determine whether this result was mediated through the antiinflammatory effects of nicotine. Using serum TNF-α as a marker of overall inflammation following ricin exposure, we determined whether nicotine treatment reduced the proinflammatory effects of ricin.

Treatment with ricin (50 µg/kg) for 14 h increased serum TNF-α levels tenfold compared with vehicle treatment (Figure 2). Treatment with nicotine (0.4 µg/kg) 2 h after ricin exposure significantly reduced the serum TNF-α levels

by 50% (Figure 2). Nicotine alone had no effect on serum TNF-α levels (Figure 2).

Nicotine Protects against Ricin-Mediated Organ Dysfunction

Ricin exposure has previously been shown to cause significant organ damage, particularly in the kidney (5,6) and the liver (26). This ricin-mediated damage has been linked to its proinflammatory actions and cytokine release leading to a proinflammatory transcriptional response in the target organs, increased oxidative stress, and subsequent cellular dysfunction. Because nicotine significantly attenuated the increase in serum proinflammatory cytokines as assessed by TNF-α levels following ricin exposure, we hypothesized that nicotine may also protect against ricin-induced organ damage. Following ricin exposure, using serum markers of organ function, we focused on kidney (BUN and creatinine) and liver (ALT), the two primary

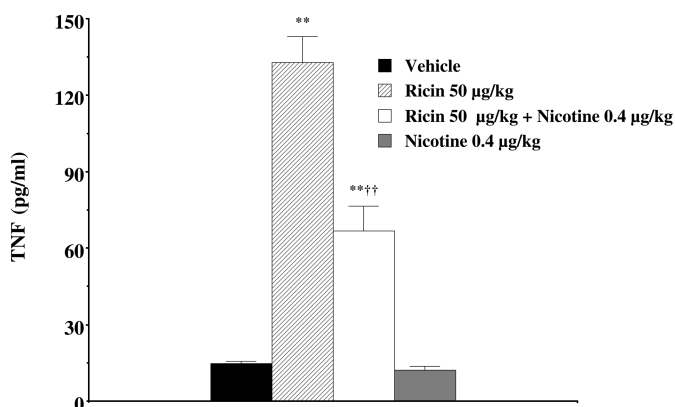


Figure 2. Nicotine attenuates ricin-mediated increase in serum TNF- α levels. Exposure of mice to ricin (50 $\mu\text{g}/\text{kg}$) for 14 h increased serum TNF- α levels, an effect reduced by nicotine (0.4 $\mu\text{g}/\text{kg}$) treatment. Nicotine alone had no effect on serum cytokine levels. Results are mean \pm SEM ($n = 10$), ** $P < 0.01$ versus vehicle treated mice, †† $P < 0.01$ versus ricin treated mice.

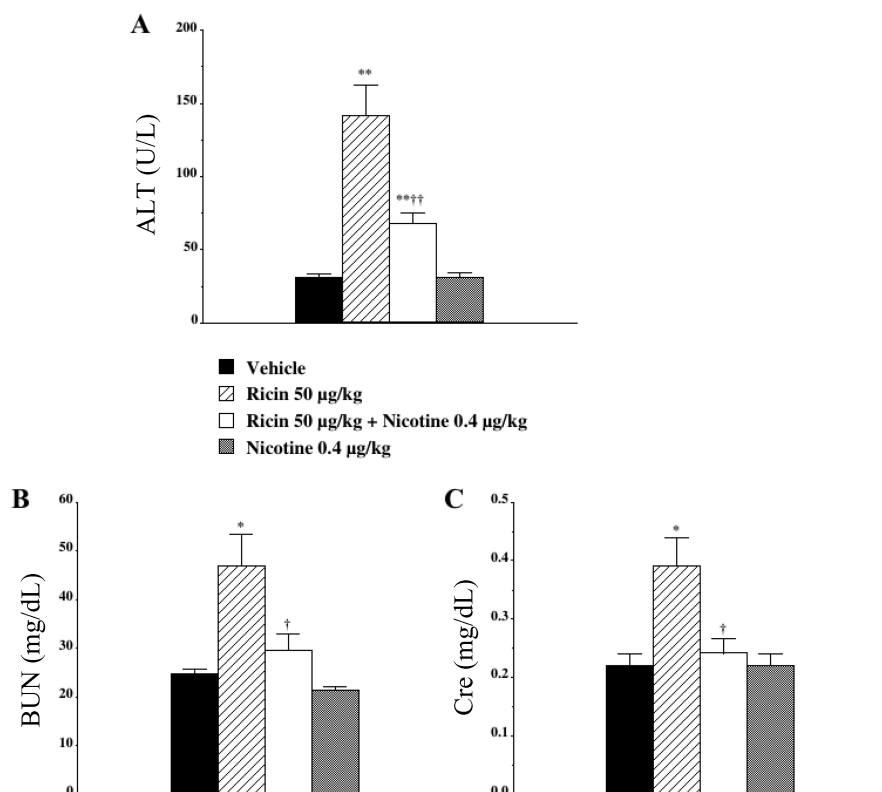


Figure 3. Ricin-mediated liver (A) and kidney (B and C) damage is reduced by nicotine treatment. Exposure of mice to ricin (50 $\mu\text{g}/\text{kg}$) for 14 h resulted in increased serum levels of ALT (A), BUN (B), and creatine (Cre) (C), demonstrating significant liver and kidney dysfunction, effects attenuated by nicotine treatment. Nicotine alone had no effect on serum levels of ALT, BUN or Cre. Results are mean \pm SEM ($n = 10$), * $P < 0.05$, ** $P < 0.01$ versus vehicle-treated mice, † $P < 0.05$, †† $P < 0.01$ versus ricin-treated mice.

organs affected by ricin, to determine whether nicotine was able to maintain their function following exposure. Additionally, following ricin exposure oxidative stress in the liver and kidney, as measured by tissue MDA levels, was determined and again any protective effect of nicotine assessed.

Exposure to ricin (50 $\mu\text{g}/\text{kg}$) for 14 h resulted in multiple organ failure with significantly increased serum levels of ALT (Figure 3A), BUN (Figure 3B), and creatine (Figure 3C), indicating liver and kidney damage. Nicotine (0.4 $\mu\text{g}/\text{kg}$) administration 2 h after ricin exposure attenuated the rise in serum levels of ALT, BUN, and creatine following ricin treatment (Figure 3). Liver and kidney levels of MDA, a marker of oxidative stress, were increased following 14-h exposure to ricin (50 $\mu\text{g}/\text{kg}$) (Figure 4). The rise in liver MDA levels following ricin treatment was attenuated by nicotine (0.4 $\mu\text{g}/\text{kg}$) treatment (Figure 4), and the rise in kidney MDA levels was completely prevented by nicotine treatment (Figure 4).

DISCUSSION

Our results have demonstrated that nicotine reduces organ failure and improves mouse survival following ricin exposure. The protective effect of nicotine appears to be associated with its anti-inflammatory effect, suggesting a possible therapeutic strategy of activating the cholinergic anti-inflammatory pathway following ricin exposure to protect against multiple organ failure. Nicotine treatment reduced levels of the inflammatory cytokine TNF- α and improved both liver and kidney function while reducing the oxidative stress observed in these organs following ricin exposure. The overall effect of nicotine on maintaining liver and kidney function while reducing systemic inflammation may account for the reduced mortality observed with ricin exposure.

Ricin has been shown to induce a severe inflammatory response that has been linked to development of acute renal failure (5,6). Ricin exposure also

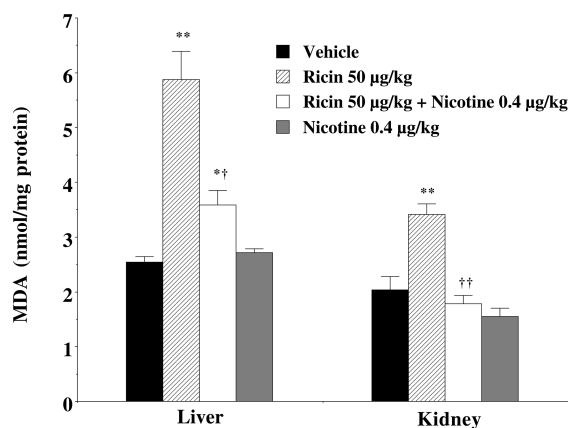


Figure 4. Ricin-induced increases of oxidative stress in liver and kidney and protection by nicotine. Exposure of mice to ricin (50 µg/kg) for 14 h resulted in significantly increased liver and kidney MDA levels indicative of oxidative stress. Nicotine partially prevented the increased oxidative stress in the liver while completely protecting the kidney from ricin-mediated increases in oxidative stress. Nicotine alone had no effect on oxidative stress in either organ. Results are mean ± SEM (n = 10), **P* < 0.05, ***P* < 0.01 versus vehicle-treated mice, †*P* < 0.05, ††*P* < 0.01 versus ricin-treated mice.

results in hypoglycemia and hepatic injury, effects linked to an increased neutrophil and monocyte infiltration with a subsequent increase in inflammatory cytokine levels (26). Ricin may increase the multiple organ damage induced by inflammatory cytokines by affecting cellular antioxidant enzyme levels; ricin exposure reduces the activities of superoxide dismutase and glutathione peroxidase while increasing xanthine oxidase activity in both kidney and liver, resulting in increased lipid peroxidation products (27–30), an effect observed in this study. Human airway cells exposed to ricin show a marked inflammatory response with activation of nuclear factor (NF)-κB and increased expression of cytokines such as TNF-α (31). The resulting organ failure, which has been observed in humans following exposure to ricin (32) eventually leads to death (26,33).

The proinflammatory effects of ricin have been linked to increased stimulation of the secondary messenger systems ERK (extracellular signal-regulated protein kinase), JNK, and p38 MAPK, with resulting activation of transcription factor NF-κB, which leads to inflammatory

gene expression (2,3,31,34). These pathways are activated by ricin not only in macrophages and inflammatory cells (3,35,36) but also other cell types such as hepatocytes and lung cells (6,23,26,31). The resulting increased expression of proinflammatory chemokines and cytokines can enhance immune cell infiltration and increase the damage to specific organs, including the lung, kidney, and liver (33). Various routes of administration of ricin: intrapulmonary (6,37), intraperitoneal (26,30), oral (4, 37), and intravenous (5), have all resulted in a systemic inflammatory response and multiple organ damage, demonstrating that ricin can achieve a systemic distribution and inflammatory response.

Activation of the antiinflammatory cholinergic pathway has been demonstrated to have an antiinflammatory effect in a wide variety of disease states (38), including reducing systemic inflammation. This effect can be observed either with vagal activation (25,39) or direct administration of nicotine (16,19,20). More recently the cholinergic antiinflammatory pathway has been shown to be activated following pharmacologic cholinesterase inhibition (40,41) or appli-

cation of the acetylcholine precursor choline (42). Specific receptors for nicotine have been shown to be present on lymphocytes from humans, rats, and mice. Nicotine has been shown to have T-cell dependent and independent effects, and the antiinflammatory effects of nicotine-receptor activation have been identified as mediated through activation of the alpha7 nicotinic acetylcholine receptor (alpha7nAChR) on immune cells (42,43), with subsequent activation of jak2 and STAT 3 (44) and a resultant decrease in proinflammatory cytokine production, particularly TNF-α (42,45).

Nicotine suppresses production of inflammatory mediators such as IL-1, IL-8, and prostaglandin-E2 from human macrophages (46) and IL-2 and TNF-α from human mononuclear cells (42), an effect mediated through modulation of NF-κB activation (46). Recently, administration of the cholinesterase inhibitor physostigmine in an experimental model of sepsis was shown to reduce activation of NF-κB and suppress circulating proinflammatory cytokines (TNF-α, IL-1, and IL-6) and neutrophil infiltration (41).

The protection against ricin-mediated inflammation and systemic organ failure by nicotine is likely due to the antiinflammatory effect of nicotine-blocking NF-κB activation and inflammatory gene expression. The antiinflammatory effect of nicotine requires signaling through the alpha7nAChR, with the subsequent inhibition of NF-κB activation and suppression of the release of proinflammatory cytokines, including TNF-α, the cytokine that appears to be the major ricin-induced proinflammatory mediator. More recently nicotine has been shown to have protective effects against both the kidney and liver (47,48), organs severely damaged by ricin exposure. Nicotine pretreatment protected the kidney from ischemia-reperfusion injury, reducing glomeruli damage, neutrophil infiltration, and expression of inflammatory chemokines and cytokines including TNF-α, effects again mediated through the alpha7nAChR (47). Activation of the vagus nerve or administration of nicotine

prevents Fas-induced apoptosis in mouse liver, again through activation of the $\alpha 7nAChR$ (48). The mechanism of activation in this case is proposed to be through reduced hepatocyte production of reactive oxygen species and subsequent reduction in cellular oxidative stress (48), mechanisms of cell damage that may be activated by ricin.

Nicotine's effect on antioxidant systems is complex, with reports of decreased activities of antioxidant enzymes such as glutathione peroxidase and catalase, but nicotine has also been shown to upregulate expression of heme oxygenase-1, a stress-inducible protein that functions as an antioxidant enzyme (49), as well as superoxide dismutase levels in the kidney (50). With ricin having significant prooxidant effects (26,30,51), any protective antioxidant enzymes that may be induced by nicotine may prove effective in maintaining organ function following ricin exposure. However, it is likely that nicotine's main protective mechanism is through the cholinergic antiinflammatory pathway.

Currently there is no antidote to ricin, and the treatment options following exposure consist of providing supportive measures to maintain organ function and removing the toxin from the body. Therefore, the development of new therapies that maintain organ function and delay mortality following ricin poisoning would be invaluable both for accidental and deliberate exposure of the human population. The data presented here suggest activation of the cholinergic antiinflammatory pathway may prove to be an effective therapeutic strategy to improve survival following ricin exposure. This effect may be induced centrally, by stimulating the vagus nerve or inhibiting cholinesterases, or peripherally, using nicotine or a specific $\alpha 7nACh$ -receptor agonist.

DISCLOSURE

We declare that the authors have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the re-

sults and discussion reported in this paper.

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