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Stimulation of acetylcholine receptors impairs host defense during pneumococcal pneumonia

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Abstract

The cholinergic nervous system can inhibit the systemic inflammation accompanying sepsis by virtue of a specific action of acetylcholine on α7 cholinergic receptors. We here sought to determine the effect of nicotine, a α7 cholinergic receptor agonist, on the host response to pneumonia caused by *Streptoccocus pneumoniae*.

Mice were intranasally infected with *S. pneumoniae* and treated with nicotine or saline intraperitoneally using a treatment schedule shown to improve host defense against abdominal sepsis.

Nicotine treatment was associated with a transiently enhanced growth of *S. pneumoniae*, as indicated by higher bacterial loads in both lungs and blood at 24 hours after infection. At 48 hours after infection, bacterial burdens had increased in both treatment groups and no differences were present anymore. Remarkably, mice treated with nicotine showed enhanced lung inflammation at 24 hours after infection. Moreover, both lung and plasma concentrations of the proinflammatory cytokines tumor necrosis factor-α and interferon-γ were higher in nicotine treated animals at this time point. Additional studies examining the effect of nicotine on the immediate (4 hours) inflammatory response to *S. pneumoniae* did not reveal an anti-inflammatory effect of nicotine either.

These data suggest that nicotine transiently impairs host defense in pneumococcal pneumonia.

Keywords: airway infection, airway inflammation, cytokines and chemokines, animal

Introduction

The cholinergic nervous system can regulate inflammation via its principal neurotransmitter acetylcholine (1). In the so-called cholinergic anti-inflammatory pathway enhanced efferent activity of parasympathetic nerve endings results in the release of acetylcholine, which by a specific action on α7 nicotinic cholinergic receptors on macrophages inhibits proinflammatory cytokine production (2). Disruption of this pathway by surgical division of the vagus nerve led to enhanced release of tumor necrosis factor (TNF)-α and accelerated the development of hypotensive shock after intravenous injection of lipopolysaccharide (LPS) into rats (3). Conversely, electrical stimulation of the efferent vagus nerve prevented the development of shock and attenuated the release of TNF-α in endotoxemic rats (3, 4). Moreover, stimulation of α7 cholinergic receptors by specific agonists, such as nicotine or 3-(2,4-dimethoxybenzylidene) anabaseine (GTS-21), attenuated TNF-α release and improved survival in mice challenged with LPS (5-7) and in mice with abdominal sepsis induced by cecal ligation and puncture (CLP) (6, 7).

Recent evidence suggests that stimulation of α7 cholinergic receptors in the lung inhibits local inflammation. Alveolar macrophages and respiratory epithelial cells were reported to express α7 cholinergic receptors in the normal lung, whereas in lungs with acid aspiration induced injury expression of α7 cholinergic receptors was also detected on infiltrating neutrophils (8). In addition, systemic administration of nicotine, choline or the specific α7 agonist PNU-282987 attenuated acid aspiration induced lung injury and inflammation, as reflected by a reduction in lung vascular permeability, neutrophil influx into bronchoalveolar lavage fluid (BALF) and local TNF-α concentrations (8). Moreover, our laboratory showed that intrapulmonary delivery of the α7 agonist GTS-21 attenuated TNF-α release into BALF of

mice exposed to LPS via the airways (9). To date, knowledge of the effect of cholinergic stimulation during lung infection is limited to one study, in which nicotine administration to mice was associated with enhanced viral loads during experimental influenza infection (10). We here sought to determine the effect of nicotine on host defense against bacterial pneumonia. Such knowledge is important considering that pneumonia has a considerable impact on health care and is the leading cause of sepsis (11, 12).

Materials and methods

Mice

Pathogen-free 9 week old female C57BL/6 mice were purchased from Harlan (Horst, The Netherlands). The Institutional Animal Care and Use Committee of the Academic Medical Center approved all experiments. At the start of the experiments mice were 10 weeks old.

Induction of pneumonia and design

Pneumonia was induced as described previously (13-15). Briefly, *Streptococcus pneumoniae* serotype 3 (ATCC 6303, American Type Culture Collection, ATCC 6303, Rockville, MD) were grown for 6 hours to mid-logarithmic phase at 37°C using Todd-Hewitt broth (Difco, Detroit, MI), harvested by centrifugation at 1500xg for 10 minutes, and washed twice in sterile isotonic saline. Bacteria were resuspended in sterile isotonic saline at a concentration of $1x10^6$ colony forming units (CFUs)/ml, as determined by plating serial 10-fold dilutions on blood agar plates. After preparation of the inocula, mice were lightly anesthetized by inhalation of 2% isoflurane (Abbott Laboratories Ltd., Kent, UK) / 2 liters of O_2, and 50 µl of the bacterial suspension (containing 5×10^4 CFUs *S. pneumoniae*) was inoculated

intranasally. Mice received an intraperitoneal injection (total volume 200 µl) with either vehicle (sterile normal saline) or nicotine (Sigma, St. Louis, MO) at a dose of 400 µg/kg starting directly after infection and with 8-hourly intervals thereafter until the end of each experiment; this treatment schedule was previously shown to protect mice from death induced by either LPS or CLP induced sepsis (7).

Sample harvesting and preparation

4, 24 or 48 hours after induction of pneumonia mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium; active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Meidrecht, The Netherlands) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes. Whole lungs were harvested and homogenized in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs were determined in lungs and blood from serial dilutions plated on blood agar plates and incubated at 37°C for 16 h before colonies were counted. For cytokine measurements, lung homogenates were diluted with an equal volume of lysis buffer (pH 7.4) containing 300 mM NaCl, 30 mM Tris, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 1% Triton X-100, and AEBSF (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride), EDTA, Pepstation A, and Leupeptin (all from MP Biomedicals, Ohio, concentrations in accordance with the manufacturer's recommendations) and incubated for 30 minutes. Homogenates were centrifuged at 1500xg at 4°C for 15 minutes, and supernatants were stored at -20°C until assays were performed.

Histology

Lungs for histology were prepared and analyzed as described earlier by a pathologist (S.F.) who was blinded with respect to treatment groups (13-15). The parameters bronchitis, edema,

interstitial inflammation, intra-alveolar inflammation, pleuritis and endothelialitis were graded on a scale of 0 to 4 with 0 as ‘absent’ and 4 as ‘severe’. The total ’lung inflammation score’ was expressed as the sum of the scores for each parameter, the maximum being 24. Besides that the percentage of inflammation was determined. Granulocyte staining was done using FITC-labelled rat anti-mouse Ly-6G mAb (Pharmingen, San Diego, CA) exactly as described (14).

Assays

TNF-α, interleukin (IL)-6, interferon (IFN)-γ, IL-10 and monocyte chemoattractant protein (MCP)-1 levels were determined using a commercially available cytometric beads array multiplex assay (BDBiosciences, San Jose, CA) in accordance with the manufacturer’s recommendations. Macrophage inflammatory protein (MIP)-2 and cytokine-induced neutrophil chemoattractant (KC) were measured by ELISA (R & D Systems, Abingdon, UK).

Statistical Analysis

All values are means ± SEM. Differences between groups were analyzed by Mann Whitney *U* test. Correlations between bacterial loads and lung cytokine concentrations were calculated using the Spearman rho test. All analyses were done using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). $P<0.05$ was considered to be statistically significant.

Results

Nicotine transiently impairs antibacterial defense

To obtain a first insight in the impact of nicotine on host defense during pneumonia, we infected mice with *S. pneumoniae* and concurrently treated them with either nicotine (400 μg/kg) or normal saline every 8 hours (7). In the first experiments mice were killed 24 or 48 hours after infection and bacterial loads were determined in whole lungs and blood (Figure 1). Nicotine treatment was associated with significantly higher bacterial burdens in both lungs and blood at 24 hours post infection (both $P < 0.01$ versus saline). Bacterial loads increased in both treatment groups thereafter and no differences existed anymore at 48 hours (Figure 1).

Impact of nicotine on lung inflammation

Nicotine and other α7 cholinergic agonists have been reported to exert anti-inflammatory effects in models of sterile lung injury (8, 9). In theory such anti-inflammatory effects could impair antibacterial defense, considering that a certain extent of inflammation is required for an adequate innate immune response to respiratory pathogens (16, 17). Therefore, we determined the effect of nicotine on the pulmonary inflammation accompanying pneumonia by semi-quantitative analysis of lung histopathology (Figure 2). Nicotine treatment was associated with increased lung inflammation at 24 hours after induction of pneumonia, as reflected by an increased pathology score at this time point ($P < 0.001$ versus saline); the enhanced inflammatory response in nicotine treated animals was further illustrated by a strongly increased neutrophil recruitment into lung tissue (insets in Figures 2A and B, showing Ly-6 stainings for neutrophils). At 48 hours after infection, pathology scores did not differ anymore between groups. Since CXC chemokines have been implicated in the recruitment of neutrophils to sites of inflammation (18), we determined the local

concentrations of MIP-2 and KC in whole lung homogenates. Nicotine did not influence pulmonary MIP-2 or KC levels at 24 hours after infection; at 48 hours KC levels were lower in nicotine treated mice ($P < 0.05$ versus saline), whereas MIP-2 levels were similar in both groups (Figure 3).

Nicotine increases lung and plasma cytokine levels

To obtain a further insight into the effect of nicotine on the host inflammatory response during pneumococcal pneumonia, we measured local and systemic cytokine concentrations at 24 and 48 hours after infection (Figure 4). Nicotine treatment was associated with higher lung levels of TNF-α and IFN-γ, and higher plasma levels of TNF-α, IFN-γ and MCP-1 at 24 hours after infection (all $P < 0.05$ versus saline); at 48 hours neither lung nor plasma concentrations of these mediators differed between groups. The pulmonary and plasma levels of IL-6 and IL-10 were similar in both groups at both time points. To evaluate whether lung cytokine levels were proportionate to the bacterial load, we calculated the correlations between pulmonary bacterial loads and cytokine levels 24 hours after infection. Positive correlations were found between bacterial load and TNF-α ($r = 0.71$, $P < 0.005$), IL-6 ($r = 0.63$, $P < 0.01$) and IFN-γ ($r = 0.74$, $P = 0.001$); correlations between bacterial loads and MCP-1 or IL-10 levels were not significant.

Impact of nicotine on the early immune response

The enhanced cytokine response at 24 hours post infection in nicotine treated mice could have been the result of the higher bacterial loads in these animals, providing a more potent proinflammatory stimulus. Therefore, we wished to evaluate the influence of nicotine on the early inflammatory response to pneumonia. Mice were intranasally infected with *S. pneumoniae*, treated immediately thereafter with either nicotine (400 µg/kg) or saline and

lungs and blood were obtained 4 hours later. At this early time point bacterial loads in lungs did not differ between groups (Table I), whereas blood cultures remained sterile (data not shown). Lung concentrations of cytokines and chemokines did not differ between groups with the exception of IL-6, which were higher in nicotine treated animals ($P < 0.05$ versus saline) (Table I). Please note that overall cytokine levels were low in both groups. IFNγ levels were below detection level. Plasma cytokine levels were either very low or undetectable in both nicotine and saline treated animals and not different between groups (data not shown).

Discussion

The cholinergic anti-inflammatory pathway, mediated by the vagus nerve and α7 cholinergic receptors, has been implicated as a neuronal feedback system that serves to limit inflammatory responses (1). We and others recently demonstrated that chemical stimulation of α7 cholinergic receptors in the pulmonary compartment results in inhibition of sterile lung inflammation induced by local instillation of either LPS or acid (8, 9). We here sought to determine the effect of nicotine on the host response to respiratory tract infection caused by the most common causative pathogen in community-acquired pneumonia, *S. pneumoniae*. Our main finding was that nicotine transiently impairs host defense during pneumococcal pneumonia as reflected by higher bacterial loads in lungs and blood in mice treated with nicotine, 24 hours after induction of pneumonia. The enhanced growth of pneumococci was accompanied by increased inflammation in the lungs, as indicated by histopathology and cytokine levels.

Several lines of evidence indicate that stimulation of the vagus nerve and/or pharmacologic α7 cholinergic receptor agonists may be a useful strategy in the treatment of the severe inflammation accompanying sepsis. First, electrical stimulation of the efferent vagus nerve prevented the development of shock and attenuated the release of TNF-α in endotoxemic rats (3, 4). Second, both electrical stimulation of the vagus nerve and stimulation of α7 cholinergic receptors by specific agonists diminished systemic inflammation and improved the outcome of mice with polymicrobial abdominal sepsis (6, 7, 19). Thus far, knowledge of the impact of stimulation of α7 cholinergic receptors in pneumonia was highly limited. In light of the fact that pneumonia is the most common cause of sepsis (11), such knowledge is of great relevance for further exploration of manipulating the cholinergic anti-inflammatory pathway as a potential novel therapeutic strategy in sepsis. In this respect, especially pneumonia caused by *S. pneumoniae* is important. *S. pneumoniae* is the most prevalent microorganism in community-acquired pneumonia, responsible for more than half a million cases each year in the United States alone, bearing a fatality rate of 5-7% (20). Bacteremia with *S. pneumoniae* originates in almost 90% of cases from the lungs. In addition, in recent sepsis trials the pneumococcus was an important causative pathogen especially in the context of pneumonia (21).

Recent studies have shown that α7 cholinergic receptors are expressed in the lung and that stimulation of these receptors by various treatments attenuates lung inflammation and injury (8, 9); in one of these investigations nicotine, given as a single dose of 3.5 mg/kg, was shown to reduce pulmonary edema, lung vascular permeability, neutrophil infiltration and TNF-α and MIP-2 release via a α7 cholinergic receptor dependent mechanism in a model of acid aspiration induced pneumonitis (8). In contrast to the model of acid aspiration induced lung injury, which induces acute lung inflammation, experimentally induced pneumococcal

pneumonia is associated with a gradual onset of inflammation and the development of an innate immune response over the first 24-48 hours (13-15).. Hence, rather than giving a single high dose, we chose a nicotine dosing and treatment scheme that previously was demonstrated to reduce lethality and systemic inflammation induced by abdominal sepsis caused CLP-induced faecal peritonitis (400 μg/kg every 8 hours) (7). This dose is in the same range as used in a model of influenza pneumonia; herein, nicotine administered by miniosmotic pumps at a rate of 1-2 mg/kg per day, reduced lung inflammation as measured by histopathology (10). Moreover, a nicotine dose within the same range was reported to be effective in reducing LPS-induced systemic inflammation (7), LPS-induced uveitis (22) and renal ischemia/reperfusion injury (23). Together these data indicate that nicotine, while effective in diminishing systemic and lung inflammation in several models, is not effective in reducing lung inflammation in the clinically relevant model of pneumococcal pneumonia. The apparent discrepancy between the current study and earlier studies on the effects of nicotine may not only be related to the differences in body compartments examined (lung versus abdominal cavity/circulation), but also to the type of causing microorganism (Gram-positive versus Gram-negative). In this respect it would be interesting to study the effect of nicotine during Gram-negative pneumonia. Of note, at 24 hours after infection most parameters of inflammation (histopathology, cytokine concentrations) were exaggerated in mice treated with nicotine. This most likely was caused by the relatively increased bacterial loads (providing a more potent proinflammatory stimulus) at this time point. Indeed, this "proinflammatory" effect of nicotine was only seen at the time point where bacterial loads were higher (24 hours) and not at either an earlier (4 hours) or later time point (48 hours); in addition, the extent of inflammation in this model closely follows the bacterial burden in the lungs (17, 24), and strong correlations were found between the pulmonary bacterial load and proinflammatory cytokine concentrations in the current study. This may also explain why at 24 hours more

neutrophils were present in the lungs of mice treated with nicotine. In this respect it should be noted that neutrophils express several nicotinic receptors, including the α7 cholinergic receptor (25), and that stimulation of these receptors has been shown to inhibit rather than enhance neutrophil migration (26).

As mentioned, nicotine treatment was associated with higher bacterial loads at 24 hours after infection. Considering that an adequate early inflammatory reaction in the lung is essential for mounting an effective host defense response in this model of pneumococcal pneumonia (17), we speculated that nicotine might inhibit the innate immune response very early after infection and thereby facilitate subsequent bacterial growth. However, such an effect of nicotine could not be shown at 4 hours after infection with *S. pneumoniae* (Table I). An alternative explanation could be a relatively defective phagocytosis and/or killing of *S. pneumoniae* in the presence of nicotine. Indeed, nicotine has been reported to enhance the replication of *Legionella pneumophila* in cultures of mouse alveolar macrophages, which was associated with inhibition of the production of proinflammatory cytokines such as TNF-α and IL-12 (27).Moreover, exposure of human neutrophils to nicotine reduced their ability to kill several oral bacterial pathogens (28). Our laboratory is currently examining in detail the effect of various cholinergic receptor agonists on antibacterial effector functions of different leukocyte subsets.

Although our study did not examine the effect of inhaled nicotine during pneumococcal pneumonia, it is interesting to note that smokers and even nonsmokers exposed to second hand smoke have an increased risk for invasive pneumococcal infection (29). A causative role for smoking in the pathogenesis of pneumococcal pneumonia_is plausible, but has not been directly demonstrated. Clearly, smoking damages local immune defences within the airways

and can enhance the binding of *S. pneumoniae* to pharyngeal cells (30). We here add to this that parenteral nicotine exposure transiently facilitates the growth of pneumococci in the lungs of mice experimentally infected with this pathogen.

In conclusion, we show that nicotine, given at a dose and treatment schedule that was earlier found to protect mice from death due to CLP-induced polymicrobial sepsis (7), transiently impairs antibacterial defense during pneumococcal pneumonia. Nicotine was unable to inhibit the inflammatory response to *S. pneumoniae* in the lung or the circulation: one day after infection, when bacterial loads were increased in nicotine treated mice, pulmonary and systemic inflammation were even enhanced.

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Table 1

Effect of nicotine on the very early (4 hours) host response to pneumococcal pneumonia

Measurements in lungs	Saline	Nicotine
Bacterial load (10 log/ml)		
CFU	4.03 ± 0.09	4.14 ± 0.07
Cytokines/Chemokines (pg/ml)		
MIP-2	9842 ± 702	11460 ± 907
KC	4023 ± 46	4426 ± 680
TNF-α	67.4 ± 7.5	63.0 ± 8.3
IL-6	18.0 ± 2.3	30.2 ± 3.7*
IL-10	26.6 ± 5.6	14.4 ± 2.4
MCP-1	455 ± 69	426 ± 101

Mice were intranasally infected with *S. pneumoniae*, and treated with either nicotine (400 μg/kg) or saline directly after infection. Mice were killed 4 hours later. Data represent mean ± SEM of 8 mice per group at each time point. * P<0.05 versus saline.

Figure 1

Nicotine treatment transiently enhances bacterial outgrowth in lung and blood. Mice were intranasally infected with *S. pneumoniae*, and treated with either nicotine (400 µg/kg; open bars) or saline (black bars) every 8 hours until sacrifice. Data represent mean ± SEM of 8 mice per group at each time point. ** $P<0.01$ versus saline.

Figure 2

Nicotine transiently enhances pneumonia induced lung pathology. Mice were intranasally infected with *S. pneumoniae*, and treated with either nicotine (400 µg/kg) or saline every 8 hours until sacrifice. Representative lung slides of saline treated (panels A and B) and nicotine treated (panels C and D) mice 24 hours (panels A and C) and 48 hours (panels B and D) after infection. H&E staining: magnification x 4. Insets: Ly-6 granulocyte stainings (magnification x 4). Pictures are representative for 8 mice per group at each time point. E:
Semi-quantitative pathology scores, determined by a scoring system described in the Methods section, 24 and 48 hours after infection. Data represent mean ± SEM of 8 mice per group at each time point. Black bars represent saline treated mice, open bars represent nicotine treated mice. *** $P < 0.001$ versus saline.

Figure 3

Lung chemokine levels. Mice were intranasally infected with *S. pneumoniae*, and treated with either nicotine (400 µg/kg; open bars) or saline (black bars) every 8 hours until sacrifice. Data represent mean ± SEM of 8 mice per group at each time point. * $P < 0.05$ versus saline.

Figure 4

Lung and plasma levels of TNF-α, IFN-γ, IL-6, IL-10 and MCP-1. Mice were intranasally infected with *S. pneumoniae*, and treated with either nicotine (400 µg/kg; open bars) or saline (black bars) every 8 hours until sacrifice. Lung levels are shown in the left panels, plasma levels in the right panels. Data represent mean ± SEM of 8 mice per group at each time point. * $P < 0.05$; ** $P<0.01$ versus saline.

Figure 1

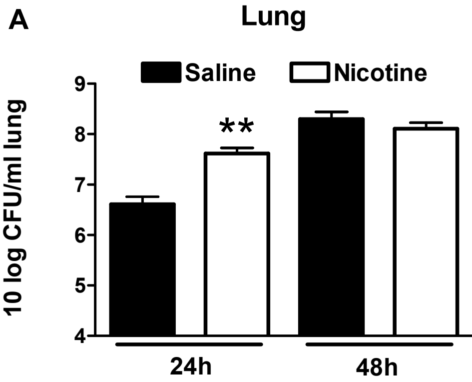


Figure 2

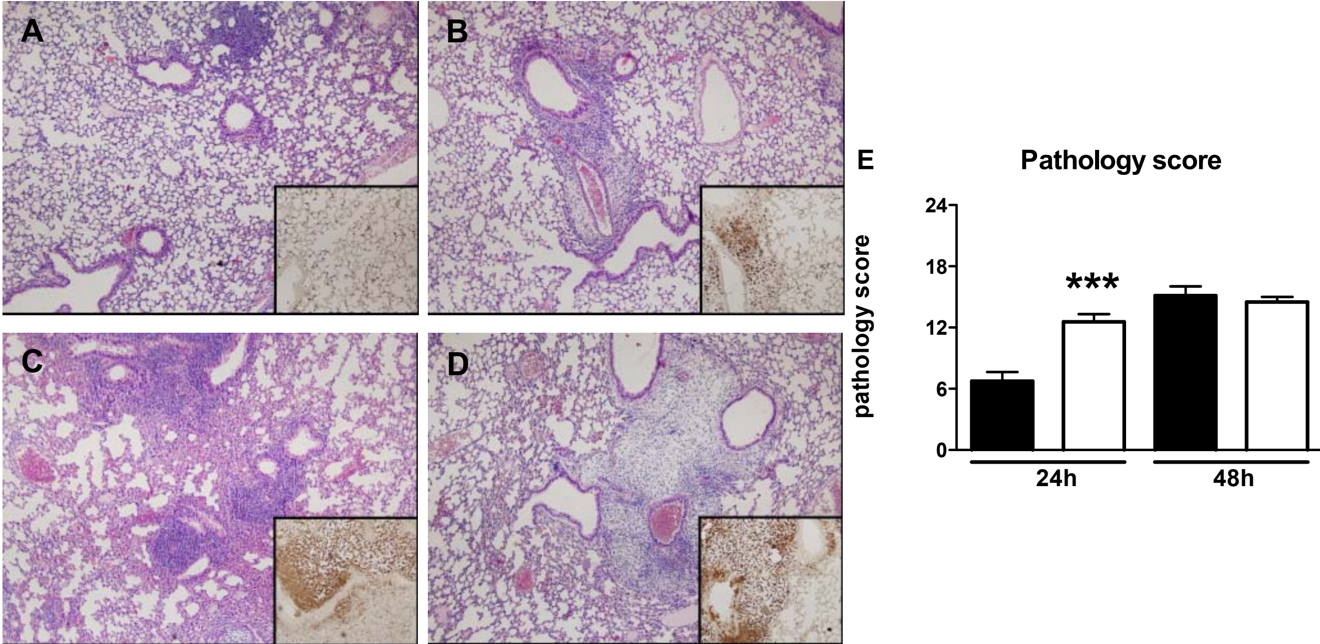
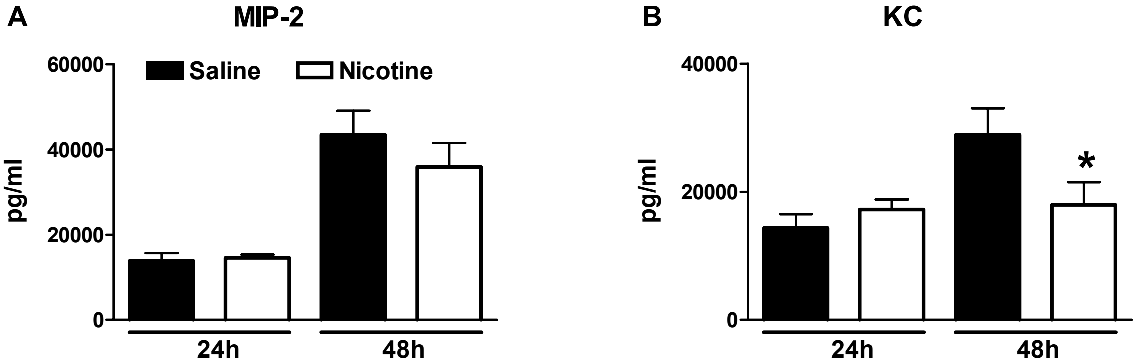


Figure 3



Lung

Plasma