Activation of Neurokinin-1 Receptors during Ozone Inhalation Contributes to Epithelial Injury and Repair

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We investigated the importance of neurokinin (NK)-1 receptors in epithelial injury and repair and neutrophil function. Conscious Wistar rats were exposed to 1 ppm ozone or filtered air for 8 hours, followed by an 8-hour postexposure period. Before exposure, we administered either the NK-1 receptor antagonist, SR140333, or saline as a control. Ethidium homodimer was instilled into lungs as a marker of necrotic airway epithelial cells. After fixation, whole mounts of airway dissected lung lobes were immunostained for 5-bromo-2'-deoxyuridine, a marker of epithelial proliferation. Both ethidium homodimer and 5-bromo-2'-deoxyuridine-positive epithelial cells were quantified in specific airway generations. Rats treated with the NK-1 receptor antagonist had significantly reduced epithelial injury and epithelial proliferation compared with control rats. Sections of terminal bronchioles showed no significant difference in the number of neutrophils in airways between groups. In addition, staining ozone-exposed lung sections for active caspase 3 showed no apoptotic cells, but ethidium-positive cells colocalized with the orphan nuclear receptor, Nur77, a marker of nonapoptotic, programmed cell death mediated by the NK-1 receptor. An immortalized human airway epithelial cell line, human bronchial epithelial-1, showed no significant difference in the number of oxidant stress-positive cells during exposure to hydrogen peroxide and a range of SR140333 doses, demonstrating no antioxidant effect of the receptor antagonist. We conclude that activation of the NK-1 receptor during acute ozone inhalation contributes to epithelial injury and subsequent epithelial proliferation, a critical component of repair, but does not influence neutrophil emigration into airways.

Keywords: oxidant airway injury; neutrophil emigration; cell proliferation; neurokinin-1 receptor; nonapoptotic programmed cell death

Ozone, a major component of photochemical air pollution, continues to be a significant public health concern. The acute inhalation of ozone initiates a cascade of events that begins with the reaction of ozone with components of the fluid lining the respiratory tract. Resulting ozone reaction products in turn induce an oxidant stress on the underlying airway epithelium that eventually leads to the death of ciliated cells in the large conducting airways and terminal bronchioles and type I pneumocytes in the distal terminal bronchioles and proximal alveolar ducts (1). Early in this cascade, oxidant-stressed epithelial cells express and release numerous mediators. These mediators include chemotactic molecules (2) that lead to a predominately

CLINICAL RELEVANCE

This study demonstrates the role that the release of substance P and its subsequent binding to neurokinin-1 receptor on oxidant-stressed cells and progenitor cells within the airway epithelium play in orchestrating ozone-induced injury and subsequent proliferative repair.

neutrophilic influx that peaks 8 to 12 hours after exposure (3, 4). Neutrophilic inflammation is followed by epithelial repair, inclusive of epithelial cell proliferation with airway basal cells and Clara cells proliferating to replace lost ciliated cells in the proximal conducting airways and terminal bronchioles, respectively (5, 6). Type II pneumocytes are the progenitor cells for the lost type I cells in the distal terminal bronchioles and proximal alveolar ducts (7).

Data from humans and animals indicate that ozone inhalation induces the early release of cyclooxygenase products from airway epithelial cells (8, 9). In turn, cyclooxygenase products activate C fibers, small, nonmyelinated sensory nerves originating in the vagus nerve and terminating beneath and within the airway epithelium and around submucosal bronchial glands and blood vessels (10, 11). C fibers induce central nervous system-mediated reflexes and also release neuropeptides upon activation, including substance P, neurokinin (NK) A, and calcitonin gene-related peptide (12). Substance P has been shown to be increased in human nasal mucosa and airways after exposure to ozone (13, 14).

Upon release, substance P binds primarily to NK-1 receptors (NK-1R) (15). In turn, NK-1 receptors have been shown to be present in numerous lung structures, including the airway epithelium from the bronchi to the alveoli of guinea pigs and humans (16, 17). Substance P, via NK-1 receptors, plays a role in regulating airway blood flow, airway smooth muscle responses, airway inflammation (18), and epithelial migration and proliferation of cells, including tracheal epithelial cells, after injury (19–21). Substance P induces superoxide production in neutrophils, is a chemotactic agent for human neutrophils, and primes neutrophils for response to other activating agents (22–24). In addition, NK-1 receptor activation has been shown to initiate nonapoptotic cell death of type I alveolar cells (25).

Using neonatal capsaicin treatment, a nonselective method of C fiber ablation and NK depletion, we have previously shown that airway C fibers modulate ozone-induced injury and repair of the distal airways. In a series of experiments, we found that rats depleted of C fibers with neonatal capsaicin treatment did not develop reflex rapid, shallow breathing, epithelial injury of terminal bronchioles was greater, and there was an uncoupling of neutrophil accumulation and other inflammatory responses from repair after ozone exposure. That is, for a given amount of epithelial necrosis, C fiber ablation had no influence on the degree of inflammation, but did modulate epithelial cell turnover and proliferative repair (26, 27). Although clearly implicating

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airway C fibers and the neuropeptides they release in ozone-induced epithelial injury and repair, interpretation of these observations are complicated by the fact that we do not know how the neonatal ablation of airway C fibers with capsaicin affects the ontogeny of the distal airway epithelium and its response to oxidant stress. In addition, neonatal capsaicin treatment is non-selective, and does not permit us to determine the role of a single neuropeptide or its receptor in ozone-induced injury and repair.

To overcome the limitations in our previous studies, and to determine the specific role that the NK-1 receptor plays in airway epithelial injury and the proliferative phase of repair after acute ozone inhalation, we administered a selective NK-1 receptor antagonist (SR140333) to rats and exposed them to 1 ppm ozone for 8 hours. Previous work in our laboratory with this model of ozone exposure in the rat has demonstrated that an 8-hour exposure to 1 ppm ozone, with an 8-hour post-exposure time in filtered air, is ideal to study epithelial injury, repair, and neutrophil influx (28, 29). SR140333 has been demonstrated to be a potent and selective NK-1 receptor inhibitor in rats, where it competitively inhibits substance P activity (30). We labeled airway epithelium with ethidium homodimer (a marker of necrotic cells) and 5-bromo-2'-deoxyuridine (BrdU; a marker of epithelial proliferation) and sampled sites along short and long airway paths using previously described techniques (31). BrdU has been used reliably and extensively in our laboratory, and is an excellent cumulative marker of epithelial proliferation. In addition, we examined the effect of NK-1 receptor antagonism on ozone-induced airway neutrophilic influx by quantifying the number of neutrophils present in bronchoalveolar lavage (BAL) fluid and airway tissue. Our hypothesis was that blocking NK-1 receptors would inhibit epithelial proliferation after ozone exposure without affecting epithelial injury or neutrophil influx.

MATERIALS AND METHODS

Animals and Exposure

Male Wistar rats were obtained from a specific pathogen-free colony (Charles River Laboratories, Kingston, NY). Rats were immediately housed in clean stainless steel chambers upon arrival at our facility and allowed to acclimate in the chambers for at least 1 week before the start of the exposure. All protocols described were approved by the University of California, Davis, Office of Environmental Health and Safety, which is responsible for the proper care and use of experimental animals.

Role of Substance P in Epithelial Injury and Repair

Rats were randomly divided into one of three groups ($n = 8$ per group): (1) ozone + NK-1 receptor antagonist; (2) ozone + saline control; or (3) filtered air + NK-1 receptor antagonist. On the morning of exposure, miniosmotic pumps (ALZET pump model 2001D, nominal pumping rate 8.0 μ l/hour; ALZA Corp., Palo Alto, CA) were primed for 3 hours with either the NK-1 receptor antagonist (SR 140333; Sanofi-Synthelabo, Paris, France) or sterile saline. The pumps were outfitted with a polyethylene tubing catheter (inside diameter, 0.76 mm; outside diameter, 1.22 mm; Intramedic; Clay Adams, Parsippany, NJ) that delivered the compound intravenously into the right jugular vein. The NK-1 receptor antagonist (0.5 mg/ml) was delivered to the animal at 200 nM/kg diluted in sterile saline.

Rats were removed from the stainless steel exposure chamber in which they were housed and anesthetized intraperitoneally with a mixture of ketamine (100 mg/ml) and xylazine (20 mg/ml). Using sterile surgical techniques, two incisions were made: one between the shoulder blades, and one ventrally above the right jugular vein. The jugular vein was dissected out to allow for cannulation. Using blunt dissection, the skin was separated from the underlying dermis on the dorsal aspect of the torso and also circumferentially around the right

side of the neck down to the incision made superior to the external jugular vein. The pump containing the antagonist or saline was placed subcutaneously between the shoulder blades, and the cannula was fed subcutaneously around the right side of the neck and then inserted into the external jugular vein. The cannula was advanced just proximal to the superior/inferior vena cava junction and held in place with silk sutures. Additional miniosmotic pumps (ALZET pump model 2ML2, nominal pumping rate 5.0 μ l/hour; ALZA Corp.) were loaded with 30 mg/ml BrdU dissolved in 0.01 N NaOH and primed in 0.9% saline overnight before surgery. The BrdU miniosmotic pump was placed subcutaneously between the shoulder blades alongside that containing the receptor antagonist or saline. Incisions were closed with 7.5 mm stainless steel wound clips (Roboz Surgical Instrument Co., Inc., Gaithersburg, MD).

Rats were allowed to recover in the exposure chambers until the beginning of exposure. One hour before the beginning of exposure, each rat received a booster of 200 nM/kg of the antagonists delivered in 200 μ l saline SQ. Control rats received 200 μ l saline SQ. Approximately 12 hours after surgery, the rats were exposed to either 1 ppm ozone or filtered air for 8 hours, followed by an 8-hour postexposure recovery in filtered air, and remained conscious throughout the exposure as previously described (4).

Before the start of the study, two groups of rats (sham treated, $n = 4$; SR140333 treated, $n = 4$) were exposed to the same ozone and treatment regimens with continual monitoring of breathing pattern to determine if the antagonists reduced respiratory frequency or increased tidal volume (V_T) and, therefore, lead to an altered distribution of ozone uptake within the airway (32). In brief, whole-body plethysmography was used to measure respiratory frequency and estimate V_T as previously described (31).

Immediately after the ozone exposure, rats were deeply anesthetized with 4% sodium pentobarbital, and the trachea was cannulated. After the chest was opened, 0.6 mM ethidium homodimer (Molecular Probes, Eugene, OR) was instilled in the lungs with gentle pressure until the lungs were just inflated. After a 15-minute incubation time in the dark, the remaining ethidium homodimer was aspirated out, and the lungs were lavaged once with warm 0.9% NaCl in water via the tracheal cannula. A cytospin sample was stained for a cellular differential of the BAL fluid. Lungs were then fixed *in situ* with a buffered zinc-formalin fixative (Z-fix; Anatech, Battle Creek, MI) via intratracheal instillation at 30 cm water pressure. Lungs and duodenum were stored in the same fixative for at least 24 hours before processing.

To quantify the number of proliferating epithelial cells, the left lung lobes were dissected to expose the airways, and whole mounts were stained for BrdU as previously described (31). Stained whole mounts of duodenum served as positive control tissue. Investigators, blinded to the treatment group, used a stereomicroscope at $\times 150$ magnification to quantify the number of ethidium- and BrdU-positive cells at nine sites along the airway tree. The positive cells were squamated and lined the airways, consistent with epithelial cells. Their epithelial morphology was subsequently confirmed with light microscopy. Five distinct airway generations were sampled: central airway, short-path proximal airway (SP PA), short-path terminal bronchiole (SP TB), long-path proximal airway (LP PA), and long-path terminal bronchiole (LP TB), as shown in Figure 3. Counts were expressed as number of positive cells per average surface area of counting field (no./mm²). Surface area of airways was estimated by measuring heights, widths, and angles of all the airway sites in rats, followed by trigonometric calculations, as described previously (31).

Neutrophils in TBs

After whole mounts of the left lung were counted, the SP TB and LP TB from each rat were paraffin embedded. Sections (7 μ m) were cut and stained with an FITC conjugate of a polyclonal rat neutrophil antibody (Accurate Chemical, Inc., Buffalo, NY). Briefly, sections were dewaxed and rehydrated following standard protocols. The sections were incubated for 30 minutes in PBS plus 6% BSA at room temperature to block any nonspecific staining. Next, sections were incubated overnight at 4°C with a 1:100 dilution of the rat neutrophil antibody in PBS with 1% immunohistochemical-grade BSA (Vector Laboratories, Burlingame, CA) or PBS plus 1% BSA only as a negative control. The

Figure 1. Immunohistochemistry for neurokinin (NK)-1 receptor in conducting airways and terminal bronchioles of a rat exposed to filtered air and 1 ppm ozone. The distal conducting airway and terminal bronchiole from a filtered air-exposed rat shows immunoreactivity of the airway epithelium and vascular smooth muscle (A) compared with the negative control (B). Similarly, a rat exposed to 1 ppm ozone for 8 hours shows immunoreactivity in the airway epithelium and vascular smooth muscle (C) compared with the negative control (D). Scale bar = 50 μ m.

next morning, sections were rinsed in PBS and mounted with Vectashield (Vector Laboratories). One section from each airway of each rat was viewed on an epifluorescent microscope (MZ FLIII stereo fluorescent microscope; Leica, San Jose, CA). A semiquantitative grading scheme was used to estimate the number of neutrophils within the epithelium and surrounding interstitium. Each section was given a score quantifying the number of neutrophils. Scores were based on a 1-to-5 grading scheme, where: 1 = no neutrophils; 2 = 1–20 neutrophils; 3 = 21–40 neutrophils; 4 = 41–60 neutrophils; 5 = more than 60 neutrophils.

Distribution of NK-1 Receptor in Airway Epithelium

Lung lobes from rats exposed to 1 ppm ozone and filtered air were stained for the presence of NK-1 receptor. Briefly, endogenous peroxidase was blocked with 0.03% hydrogen peroxide (H_2O_2) in 100% methanol for 30 minutes at room temperature. For nonspecific blocking, sections were incubated with 10% goat serum in PBS for 30 minutes at room temperature. The goat polyclonal antibody to NK-1 receptor N terminus (clone N-19; Santa Cruz Biotechnology, Santa Cruz, CA) was incubated at 1:100 to 1:250 overnight at 4°C. The antibody preincubated with a blocking peptide (Santa Cruz Biotechnology) was used as a negative control. The following day, all sections were incubated with a biotinylated goat anti-rabbit antibody (Vector Laboratories) at 1:400 for 30 minutes at room temperature. Streptavidin-horseradish peroxidase (HRP) antibody (Invitrogen, Carlsbad, CA) at 1:500 was added for 30 minutes at room temperature to all sections. The slides were developed with 3-amino-9-ethylcarbazole (AEC; Invitrogen), according to the manufacturer's protocol, and counterstained with Meyer's hematoxylin (Sigma, St. Louis, MO).

Active Caspase-3 Immunohistochemistry of Terminal Bronchioles

After whole mounts of the left lung were counted, the SP TB and LP TB from each rat were paraffin embedded. Sections (7 μ m) sections were cut, paraffin removed, and rehydrated routinely. Endogenous peroxidase was blocked with 0.03% H_2O_2 in 100% methanol for 30 minutes at room temperature. For nonspecific blocking, sections were incubated with 10% goat serum in PBS for 30 minutes at room temperature. Immediately afterwards, the blocking solution was decanted and sections were incubated in a polyclonal active caspase 3 antibody (Cell Signaling, Danvers, MA) at 1:500 in PBS overnight at 4°C. Negative control sections were incubated in PBS only with no primary antibody. Rat jejunum served as positive control tissue. The following day, all sections were incubated with a biotinylated goat anti-rabbit antibody (Vector Laboratories) at 1:400 for 30 minutes at room temperature. Streptavidin-HRP

antibody at 1:500 was added to all sections for 30 minutes at room temperature. The slides were developed with AEC according to the manufacturer's protocol, and counterstained with Meyer's hematoxylin.

Figure 2. Tidal volume (V_t) (A) and breathing frequency (B) responses in rats exposed to 1 ppm ozone for 8 hours and treated with the neurokinin (NK)-1 antagonist (squares) or vehicle (triangles). There was a significant decrease in V_t and increase in breathing frequency compared with preexposure baseline and filtered air controls (data not shown) ($P < 0.05$). There was no difference between the NK-1 receptor antagonist and vehicle parameters exposed to ozone.

Figure 3. A schematic diagram of the left lung lobe of a rat showing the short-path and long-path sample points with numbers of ethidium homodimer- and BrdU-positive epithelial cells/mm² for the central airway, proximal airways, and terminal bronchioles. There was a reduction of epithelial injury and proliferation in rats treated with the NK-1 receptor antagonist at each airway generation. There were significantly fewer ethidium homodimer-positive cells at the terminal bronchioles in rats treated with SR140333 ($n = 8$) compared with vehicle-treated rats after ozone exposure ($n = 10$) and filtered air-exposed rats administered SR140333 ($n = 4$). Significantly fewer BrdU-labeled epithelial cells were present in the proximal airways and terminal bronchioles of rats treated with the NK-1 receptor antagonist ($n = 8$) compared with vehicle-treated rats after ozone exposure ($n = 10$) and filtered air-exposed rats administered SR140333 ($n = 4$). Data points represent mean number of cells/mm² $\pm$ SE. * $P < 0.0001$ compared with all other groups. CA = central airway; LP = long path; PA = proximal airways; SP = short path; TB = terminal bronchioles.

Nur77 Immunohistochemistry of Terminal Bronchioles

To assess Nur77 colocalization with ethidium homodimer-positive cells, rats were exposed for 8 hours to 1 ppm ozone, followed by an 8-hour postexposure period in filtered air. After instillation of ethidium homodimer into their airways, as previously described, the right middle lung lobe was cannulated and fixed in 1% paraformaldehyde. The lobe was dissected to expose the airways, embedded in paraffin, and sectioned. After rehydration, the sections were coverslipped with 90% glycerol in PBS, and airways were mapped and imaged for ethidium homodimer-positive cells (BX61 microscope; Olympus, Center Valley, PA). After imaging, coverslips were removed, and sections were immersed in PBS and stained for Nur77. Briefly, endogenous peroxidase was blocked with 0.03% H₂O₂ in 100% methanol for 30 minutes at room temperature. For nonspecific blocking, sections were incubated with 10% donkey serum in PBS for 30 minutes at room temperature. The sections were incubated in 1:50 dilution of Nur77 antibody (Santa Cruz Biotechnology) overnight at 4°C. Sections of rat brain were used as positive control, and rabbit IgG (Vector Laboratories) at the same concentration as that of the primary antibody was

used as a negative control. The next day, all sections were incubated with a biotinylated donkey anti-rabbit antibody (Vector Laboratories) at 1:400 for 30 minutes at room temperature. Streptavidin-HRP antibody at 1:500 was added for 30 minutes at room temperature to all sections. The slides were developed with AEC according to the manufacturer's protocol, and counterstained with Meyer's hematoxylin. We then relocated the same airways and ethidium-positive cells using the map and imaged the same areas for Nur77. The two images were scaled and transformed to overlap by aligning similar structures (alveoli and blood vessels). The ethidium and AEC signals were blended in Adobe Photoshop (Adobe Systems Inc., San Jose, CA) with the use of "apply image" to show AEC signal in the ethidium-positive nuclei.

Oxidant Stress in Human Bronchial Epithelial Cells after NK-1 Receptor Antagonist Treatment

An immortalized human airway epithelial cell line, human bronchial epithelial (HBE)-1, was maintained in serum-free Dulbecco's modified Eagle medium/Ham's F12 with insulin (5 μ g/ml), transferrin (5 μ g/ml),

epidermal growth factor (10 ng/ml), dexamethasone (0.1 μ M), cholera toxin (10 ng/ml), and bovine hypothalamus extract (15 μ g/ml). HBE cells were plated on chamber coverslips (Fisher Scientific, Pittsburgh, PA) and grown to confluence. Before the start of the experiment, monolayers of cells were loaded with 1 μ M of the oxidant stress-sensitive dye, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (Molecular Probes) for 20 minutes at 37°C in the dark. Subsequently, the monolayers were treated with 0, 1 μ g/ml, 4 μ g/ml, or 7 μ g/ml of the SR 140333 NK-1 receptor antagonist for 30 minutes before oxidant exposure. H₂O₂ was used as the oxidant, and each concentration of NK-1 receptor antagonist was treated with either 0, 400, or 800 μ M H₂O₂. The cells were imaged using a DeltaVision microscope during oxidant stress in the presence of the inhibitor while being kept at 37°C. Using stratified random sampling, 10 fields at $\times 40$ magnification were imaged for each condition. Oxidant stress-positive and oxidant stress-negative cells were counted using Stereology Toolbox (Davis, CA).

Statistical Analysis

For the receptor antagonist study, separate ANOVA tests were performed for the ethidium homodimer and BrdU data at each airway generation (central airway, SP PA, SP TB, LP PA, and LP TB). If there was significance indicated by the ANOVA test, an *ad hoc* test, the Fisher's least-significant-difference test, was performed to compare individual groups (Systat 10.0; SPSS Inc., Chicago, IL). Before determining if there were significant between-group differences in the numbers of neutrophils in lavage, an ANOVA followed by a Bonferroni comparison test was performed between all the ozone-exposed groups, and a Student's *t* test was performed between the two filtered air-exposed groups. Because there were no significant differences shown by these tests, the number of neutrophils in lavage was compared between the ozone-exposed groups and filtered air-exposed groups using Student's *t* test (Systat 10.0). Kruskal-Wallis one-way ANOVA was performed to determine if there were significant differences in neutrophil scores between rats exposed to ozone and treated with either of the receptor antagonists and saline. For the oxidant stress experiment, an overall ANOVA showed significance and was followed by a Fisher's least-significant-difference test to compare individual groups. Data points for all results represent mean values $\pm$ SE. Results were considered significant if $P \geq 0.05$.

RESULTS

Distribution of NK-1 Receptors in Rat Lung

Using immunohistochemistry, we assessed the distribution of NK-1 receptors in rat lungs exposed to 1 ppm ozone and filtered air. Figure 1 demonstrates positive staining of the conducting and terminal bronchiole epithelium in rats. Additionally, we obtained positive staining of the smooth muscle in pulmonary arterioles.

Breathing Pattern Response to the NK-1 Receptor Antagonist

Ozone induced a significant decrease in V_T and increase in breathing frequency compared with preexposure baseline and filtered air control tissues ($P < 0.05$). There were no differences in V_T (Figure 2A), breathing frequency (Figure 2B), or minute ventilation (data not shown) between treatment groups exposed to ozone. As a result of these findings, we exposed the majority of the rats studied in large exposure chambers capable of housing several rats at one time.

Diminished Epithelial Injury and Proliferation in Rats Treated with the NK-1 Receptor Antagonist

Rats were treated systemically with an NK-1 receptor antagonist to elucidate the role of substance P in airway epithelial injury and repair after ozone exposure. Immediately after the postexposure period, rats were anesthetized and ethidium homodimer-1 was instilled into their lungs through a tracheal cannula. Ethidium homodimer readily crosses cells with disrupted cell membranes. After fixation and airway dissection,

ethidium-positive cells were expressed as number per surface area of airway.

As shown in Figure 3A, in the terminal bronchioles of rats exposed to ozone, there were significantly fewer ethidium-positive cells in the rats treated with the NK-1 receptor antagonist compared with the rats treated with the saline vehicle ($P < 0.02$).

A cumulative label of BrdU, a thymidine analog, was used as a marker of epithelial proliferation and repair. BrdU-positive epithelial cells were also expressed as number per surface area of airway.

As shown in Figure 3B, among the groups exposed to ozone, there was significantly reduced epithelial cell proliferation in rats treated with the NK-1 receptor antagonist compared with rats treated with the saline vehicle in the proximal airway (SP PA + LP PA; $P < 0.0002$) and terminal bronchioles (SP TB + LP TB; $P < 0.0001$).

NK-1 Receptor Antagonist, SR140333, Does Not Significantly Alter Neutrophil Emigration

To determine whether substance P influenced neutrophil migration into the airways after ozone exposure, the percentage of neutrophils in BAL was compared between the groups. The only significant difference in neutrophil numbers in BAL fluid was between ozone-exposed rats and filter air-exposed rats (Figure 4A). There was no significant difference in the percentage of neutrophils in the lavage of rats treated with SR140333 compared with vehicle and exposed to ozone. As shown in Figure 4B, there was no significant difference in the number of neutrophils between NK-1 receptor antagonist- or vehicle-treated groups exposed to ozone in the terminal bronchioles of the tissue sections. We conclude that SR140333 did not significantly affect neutrophil emigration into airways after ozone exposure.

NK-1 Receptor Antagonist, SR140333, Does Not Function as an Antioxidant

One explanation for the diminished epithelial injury when rats were treated with the NK-1 receptor antagonist is that the antagonist is an antioxidant, effectively protecting the airway epithelium from ozone exposure. We tested this hypothesis by exposing an immortalized normal HBE cell line, HBE-1, to 0, 400, or 800 μ M H₂O₂ in the presence of either 0, 1, 4, or 7 μ g/ml NK-1 antagonist and the oxidant stress-sensitive dye, 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate. Our goal in this study was to test the antioxidant ability of SR140333 in a cell culture assay. We chose this assay and H₂O₂ as the oxidant because we have previous experience with this system in our laboratory (2). The number of oxidant stress-positive cells as a fraction of total cell number was determined for each condition. As shown in Figure 5, no significant correlation was found between the oxidant stress-positive cells and the dose of NK-1 receptor antagonist. We conclude that the NK-1 receptor antagonist did not diminish epithelial injury and subsequent proliferation by reducing the oxidant stress during ozone exposure.

Ozone Does Not Induce Epithelial Cell Caspase-Dependent Apoptosis in Terminal Bronchioles

To determine whether a decrease in epithelial cell necrosis was associated with a change in apoptotic epithelial cells in SR 140333-treated rats, terminal bronchioles were stained with active caspase 3, a marker of apoptotic cells. As shown in Figure 6, extremely rare positive airway epithelial cells were found in rats exposed to ozone and treated with both the vehicle (Figure 6B) and NK-1 receptor antagonist, SR140333 (Figure

Figure 4. (A) Percentage of neutrophils in bronchoalveolar lavage (BAL) fluid. No significant difference was found in the percent of neutrophils in BAL fluid in rats treated with SR140333 ($n = 8$) compared with rats treated with the vehicle and exposed to ozone ($n = 10$). (B) Semiquantitative score of the number of neutrophils in terminal bronchioles in rats exposed to ozone and treated with the NK-1 receptor antagonist, SR140333 ($n = 8$), and the vehicle control ($n = 10$). No significant difference was found in the number of neutrophils within the epithelium and interstitium of airways in the two groups. Data points represent mean neutrophil (pmn) score $\pm$ SE.

6C). These cells were interpreted as incidental, and there were no significant difference in the number of apoptotic cells between the treatment groups. These results indicate that ozone induces a non-caspase-mediated form of cell death in airway epithelial cells.

Ethidium Homodimer-Positive Cells Colocalize with Nur77

Finally, to determine whether SR140333 protects airway epithelium from ozone-induced nonapoptotic programmed cell death (pcd), we investigated colocalization of ethidium homodimer-positive cells with Nur77, a key regulator of substance P-mediated nonapoptotic pcd in nonantagonist, ozone-exposed rats. Figure 7 demonstrates ethidium-positive cells with positive cytoplasmic staining for Nur77. This suggests that SR140333 is protective by blocking substance P binding to NK-1R and subsequent failure to induce nonapoptotic pcd.

DISCUSSION

In this article, we examine the role that NK-1 receptor plays in airway epithelial injury and proliferation during repair, and

Figure 5. The number of oxidant stress-positive cells over a range of H₂O₂ concentrations and doses of the NK-1 receptor antagonist ($n = 10$ for each condition). The fraction of oxidant stress-positive cells of the total cells (oxidant stress positive + oxidant stress negative) was determined. No significant difference was found in the number of oxidant stress-positive cells over the doses of SR140333. Data points represent mean number of oxidant stress-positive cells divided by the total number of cells $\pm$ SE.

whether it modulates neutrophil emigration into airways after acute ozone inhalation. First, we demonstrate that the NK-1 receptor antagonist, SR140333, did not significantly affect the rapid, shallow breathing induced by an acute ozone exposure in Wistar rats. Second, we show that treatment with the NK-1 receptor antagonist significantly attenuated epithelial cell death in terminal bronchioles, but not in proximal airways, suggesting an airway generation-specific response in ozone-induced cell death. Third, we show that treatment with the NK-1 receptor antagonist significantly attenuated epithelial cell proliferation after acute ozone exposure in both proximal and distal airways. Fourth, the NK-1 receptor antagonist did not influence the emigration of neutrophils into airways after ozone injury. Supplementing these observations, we show that: (1) airway epithelial cells expressing NK-1 receptors are distributed down the entire length of the airway; (2) ozone-induced epithelial cell death in the terminal bronchioles occurs via a nonapoptotic mechanism; (3) the NK-1 receptor antagonist, SR140333, does not attenuate cell injury by acting as an antioxidant; and (4) ethidium-positive cells in the terminal bronchioles of ozone-exposed rats are positive for the nuclear orphan receptor, Nur77. Taken together, these data indicate that substance P acting via NK-1 receptors is an important mediator in orchestrating airway epithelial cell death and proliferation after acute ozone exposure.

We found airway generational differences in the amount of epithelial cell death and proliferation in vehicle-treated rats exposed to 1.0 ppm ozone for 8 hours followed by an 8-hour postexposure period. The highest density of dead cells was found in the terminal bronchioles (Figure 3). Similar site-specific differences in ozone-induced injury and repair have been previously reported (33, 34). It has been proposed that this distribution of ozone-induced lesions is the result of numerous factors, including the distribution of ozone uptake at different airway sites, and the sensitivity of different cell types to ozone-induced injury (33). Previous work in our laboratory has shown that the pattern of distribution of ozone-induced airway injury is in part dependent upon the development of rapid, shallow breathing during exposure, which tends to protect the large conducting airways, but produces a more even distribution of

Figure 6. Immunohistochemistry for cleaved caspase 3. (A) Positive control tissue is rat small intestine, showing apoptotic enterocytes at the villous tip. (B) Ozone plus vehicle-exposed rat shows a solitary positive airway epithelial cell. (C) Ozone plus NK-1 receptor antagonist-exposed rat also shows a single positive cell in the lumen of a terminal bronchiole. Images at $\times 20$ magnification; scale bar = 50 μm . Inset images: $\times 40$ magnification; scale bar = 10 μm .

injury in terminal bronchioles (31). Subsequently, Alfaro and colleagues (32) demonstrated that rapid, shallow breathing results in a redistribution of ozone uptake, in part explaining the findings of Schelegle and coworkers. In the current study, we found that the selective NK-1 receptor antagonist SR140333, did not significantly affect ozone-induced rapid shallow breathing in a limited subsample of rats. Therefore, the observed decreases in site-specific, ozone-induced epithelial injury and proliferation were due to the direct effect of the antagonists, and not due to a change in the distribution of ozone uptake.

Figure 7. Colocalization of ethidium homodimer cells with Nur77 in rat exposed to 1 ppm ozone for 8 hours followed by an 8-hour postexposure time in filtered air. (A) White nuclei are ethidium homodimer-positive airway epithelial cells. (B) Red cytoplasmic staining demonstrates positive staining for Nur77 in the same airway. (C) Combined image demonstrating colocalization of ethidium-positive cells (white) with Nur77 (red). Scale bar = 200 μm .

Although never directly studied, it has been assumed that ozone-induced cell death of airway epithelial cells is the direct result of oxidant damage induced by ozone and/or its reaction products. Our data show that treatment with SR140333 significantly reduced the ethidium labeling in the terminal bronchioles after ozone inhalation, indicating that NK-1 receptors play some role in mediating ozone-induced cell death. Several papers have found that NK-1 receptors contribute to cellular injury and death in various animal and cell culture models of tissue injury. SR140333 reduced the epithelial ulceration and severity of colitis and infarct volume after cerebral ischemia in rats (35, 36). Castro-Obregón and colleagues (37) demonstrated, in a neuronal cell line overexpressing the NK-1 receptor, that substance P induces a nonapoptotic programmed cell death. They showed that this nonapoptotic cell death involves the recruitment of a mitogen-activated protein kinase phosphorylation cascade by the scaffold protein, arrestin 2, leading to the phosphorylation of the orphan nuclear receptor, Nur77. More recently, Lucattelli and colleagues (25) have shown that bleomycin-induced cell death of type I pneumocytes in mice is dependent upon a similar NK-1 receptor-dependent pathway. It is possible that a similar NK-1 receptor-dependent mechanism contributes to the ozone-induced cell death observed in the terminal bronchioles in our rats. We assessed the

amount of apoptosis using immunohistochemistry for active caspase 3, a key enzyme involved in the apoptotic pathway and an established marker of apoptotic cells in tissue. Terminal bronchioles in rats exposed to ozone and treated with both the NK-1 receptor antagonist and vehicle showed no significant number of apoptotic cells. Additionally we demonstrate that the ethidium-positive cells colocalize with Nur77 in ozone-exposed rats not treated with SR140333. Taken together, our results suggest that substance P, acting through the NK-1 receptor and Nur77, may contribute to epithelial cell death through a non-caspase 3-mediated mechanism.

Other mechanisms for this epithelial protective effect may exist. In this study, we investigated the possibility that SR 140333 could have direct antioxidant effects, thus protecting the epithelium from ozone-induced injury. We found no evidence of antioxidant effects when HBE cells were treated with 400 and 800 mM H₂O₂ as an oxidant in the presence of a range of SR140333 levels, including those used in rats in this study.

Another potential protective effect of SR140333 may be related to its ability to block substance P-induced vasodilation and microvascular permeability (38). Acute ozone inhalation in humans has been shown to result in the elevation in BAL fluid of several macromolecules normally restricted to the vascular compartment that may contribute to cellular injury and necrosis, including complement 3a (39). Interestingly, Park and colleagues (40) have recently demonstrated that either depleting and/or antagonizing complement in mice significantly reduces ozone-induced airway hyperresponsiveness. Several studies have also shown that NK-1 antagonists (including SR140333) blocks airway epithelial permeability after ozone exposure and airway hyperresponsiveness in other models, implicating a role of substance P in the induction of airway hyperresponsiveness as well (41, 42). Blocking microvascular permeability could limit the influx of mediators to the site of ozone-induced oxidant stress, reducing the subsequent epithelial cell death. Countering this possibility is the observation of Sertl and coworkers (43) that substance P-induced vascular permeability is limited to the large conducting airways of the rat.

Interestingly, we found a significant decrease in epithelial necrosis in the terminal bronchioles, but not in central or proximal conducting airways, of rats treated with SR140333 and exposed to ozone compared with vehicle-treated and ozone-exposed rats. This unique observation could be due to several possibilities. First, it could indicate that NK-1 receptors have a differential effect on ozone-induced injury at the different airway generations studied. This may, in part, be the result of the different cell types that are located within the proximal airways and terminal bronchioles. The epithelial populations of rat proximal bronchi are ciliated, serous, basal, and mucous cells, whereas the terminal bronchioles contain ciliated and Clara cells, and distal terminal bronchiole and proximal alveolar ducts contain type 1 and 2 pneumocytes. Although our results demonstrate that NK-1 receptors are present throughout these airways, there could be functional differences in the receptors or other mediators, which may modulate the receptors' effect on epithelial injury at these sites. Second, the delivery of SR140333 via the vasculature to the surface epithelium may differ between the airway sites studied. Third, the ethidium label is not cumulative, and we cannot rule out the possibility that epithelial injury occurs at different airway generations at different time points after ozone exposure.

SR140333 significantly attenuated ozone-induced proliferation of epithelial cells at all the sites studied. This effect is similar to the effect that substance P has on modulating the migration and proliferation of cells after the mechanical injury of tracheal epithelium (21). Substance P stimulates proliferation

and migration of guinea pig tracheal epithelial cells (19), and works synergistically with insulin-like growth factor-1 to stimulate corneal epithelial wound healing (20, 44). Vesely and colleagues found less BrdU incorporation in terminal bronchiolar epithelium of capsaicin-treated rats exposed to filtered air and ozone, suggesting that neuropeptides released by C fibers may modulate basal and reparative airway epithelial cell proliferation (27). Our observations, that the NK-1 antagonist, SR140333, decreases airway cell proliferation after ozone exposure, confirms the previous findings of Vesely and colleagues (27), and extends them to the large conducting airways examined in this study. This implies that the release of substance P from lung C fibers acts to orchestrate the proliferation of basal cells, nonciliated Clara cells, and type II pneumocytes to replace the ciliated cells and type I pneumocytes lost during and after the acute inhalation of ozone. This illustrates the need to better understand the precise mechanisms that lead to the activation of lung C fibers during acute ozone exposure. This is especially true in the terminal bronchioles, where substance P release appears to contribute to ozone-induced cell death, as well as to cell proliferation. Specifically, we propose that oxidant-stressed ciliated cells and type I pneumocytes release one or more mediators that activate lung C fibers to release substance P, and that this substance P activates cell death and proliferation pathways in the terminal bronchiolar epithelium and cell proliferation pathways in the proximal conducting airway epithelium that is in proportion to the ozone-induced oxidant stress.

Our results indicate that the NK-1 receptor antagonist did not significantly affect neutrophil emigration into airways after ozone exposure. There was no significant difference in the number of neutrophils in BAL or in terminal bronchioles of rats treated with SR140333 compared with vehicle-treated rats exposed to ozone. These observations are consistent with our previous observations in rats treated neonatally with capsaicin to ablate C fiber afferents (26). Taken together, these results suggest that substance P is not a critical mediator for neutrophil emigration into airways after ozone injury in the rat.

Within the literature, there is some discrepancy as to the importance of substance P in neutrophil function and recruitment in different experimental models. *In vitro* studies show that substance P primes and activates human neutrophils for superoxide, H₂O₂, and nitric oxide production (23, 24). In humans, substance P also appears to be important in neutrophil chemotaxis, mediating neutrophil binding to epithelial and endothelial cells and inducing release of cytokines, such as IL-8 (45). NK-1 receptor knockout mice have shown the importance of substance P in mediating neutrophil accumulation in pancreatitis and inflamed skin (46, 47).

In contrast, other papers have not substantiated the importance of substance P in neutrophil recruitment. Similar to our results with SR140333, neutrophil recruitment was not affected in a model of thermal injury in rats (48). Roch-Arveiller and colleagues examined the response of rat neutrophils to substance P and found concentration-dependent chemotaxis only at high concentrations (10⁻⁶–10⁻⁴ mol/liter) (49).

In conclusion, these observations illustrate the pivotal role that the activation of C fibers, with the subsequent release of substance P, plays in the complex cascade of events that are initiated by the acute inhalation of ozone. This role is not limited to reflex responses, such as rapid shallow breathing, but includes the modulation of cellular injury and subsequent proliferation of epithelial cells during repair. Further studies need to be conducted to examine the direct and/or indirect mechanisms that contribute to this modulation of cellular injury and repair by NK-1 receptors.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

- Plopper CG, Dungworth DL, Tyler WS. Pulmonary lesions in rats exposed to ozone: a correlated light and electron microscopic study. *Am J Pathol* 1973;71:375–394.
- Oslund KL, Miller LA, Usachenko JL, Tyler NK, Wu R, Hyde DM. Oxidant-injured airway epithelial cells upregulate thioredoxin but do not produce interleukin-8. *Am J Respir Cell Mol Biol* 2004;30:597–604.
- Hyde DM, Miller LA, McDonald RJ, Stovall MY, Wong V, Pinkerton KE, Wegner CD, Rothlein R, Plopper CG. Neutrophils enhance clearance of necrotic epithelial cells in ozone-induced lung injury in rhesus monkeys. *Am J Physiol* 1999; 277(6 pt 1):L1190–L1198.
- Vesely KR, Schelegle ES, Stovall MY, Harkema JR, Green JF, Hyde DM. Breathing pattern response and epithelial labeling in ozone-induced airway injury in neutrophil-depleted rats. *Am J Respir Cell Mol Biol* 1999;20:699–709.
- Evans MJ, Johnson LV, Stephens RJ, Freeman G. Renewal of the terminal bronchiolar epithelium in the rat following exposure to NO₂ or O₃. *Lab Invest* 1976;35:246–257.
- Puchelle E, Le Simple P, Hajj R, Coraux C. Regeneration of injured airway epithelium. *Ann Pharm Fr* 2006;64:107–113. [in French]
- Evans MJ, Johnson LV, Stephens RJ, Freeman G. Cell renewal in the lungs of rats exposed to low levels of ozone. *Exp Mol Pathol* 1976;24:70–83.
- Devlin RB, McDonnell WF, Becker S, Madden MC, McGee MP, Perez R, Hatch G, House DE, Koren HS. Time-dependent changes of inflammatory mediators in the lungs of humans exposed to 0.4 ppm ozone for 2 hr: a comparison of mediators found in bronchoalveolar lavage fluid 1 and 18 hr after exposure. *Toxicol Appl Pharmacol* 1996; 138:176–185.
- Kafoury RM, Pryor WA, Squadrito GL, Salgo MG, Zou X, Friedman M. Induction of inflammatory mediators in human airway epithelial cells by lipid ozonation products. *Am J Respir Crit Care Med* 1999;160: 1934–1942.
- Lee LY, Widdicombe JG. Modulation of airway sensitivity to inhaled irritants: role of inflammatory mediators. *Environ Health Perspect* 2001;109:585–589.
- Solway J, Leff AR. Sensory neuropeptides and airway function. *J Appl Physiol* 1991;71:2077–2087.
- Maggi CA, Giachetti A, Dey RD, Said SI. Neuropeptides as regulators of airway function: vasoactive intestinal peptide and the tachykinins. *Physiol Rev* 1995;75:277–322.
- Schierhorn K, Hanf G, Fischer A, Umland B, Olze H, Kunel G. Ozone-induced release of neuropeptides from human nasal mucosa cells. *Int Arch Allergy Immunol* 2002;129:145–151.
- Hazbun ME, Hamilton R, Holian A, Eschenbacher WL. Ozone-induced increases in substance P and 8-epi-prostaglandin F₂ alpha in the airways of human subjects. *Am J Respir Cell Mol Biol* 1993;9:568–572.
- Hokfelt T, Pernow B, Wahren J. Substance P: a pioneer amongst neuropeptides. *J Intern Med* 2001;249:27–40.
- Bai TR, Zhou D, Weir T, Walker B, Hegele R, Hayashi S, McKay K, Bondy GP, Fong T, Substance P. Substance P (NK1)- and neurokinin A (NK2)-receptor gene expression in inflammatory airway diseases. *Am J Physiol* 1995;269:L309–L317.
- O'Connor TM, O'Connell J, O'Brien DI, Bennett MW, Goode T, Burke L, Bredin CP, Shanahan F. Upregulation of neurokinin-1 receptor expression in the lungs of patients with sarcoidosis. *J Clin Immunol* 2003;23:425–435.
- Groneberg DA, Quarcoo D, Frossard N, Fischer A. Neurogenic mechanisms in bronchial inflammatory diseases. *Allergy* 2004;59: 1139–1152.
- Kim JS, Rabe KF, Magnussen H, Green JM, White SR. Migration and proliferation of guinea pig and human airway epithelial cells in response to tachykinins. *Am J Physiol* 1995;269(1 pt 1):L119–L126.
- Hara R, Katakami C, Yamamoto M. The effect of substance P with insulin-like growth factor-1 on corneal epithelial cell proliferation. *Nippon Ganka Gakkai Zasshi* 1999;103:641–646.
- Kim JS, McKinnis VS, Adams K, White SR. Proliferation and repair of guinea pig tracheal epithelium after neuropeptides depletion and injury *in vivo*. *Am J Physiol* 1997;273(6 pt 1):L1235–L1241.
- Hafstrom I, Ringertz B, Lundberg T, Palmblad J. The effect of endothelin, neuropeptide Y, calcitonin gene-related peptide and substance P on neutrophil functions. *Acta Physiol Scand* 1993;148: 341–346.
- Sterner-Kock A, Braun RK, Van der Vliet A, Schrenzel MD, McDonald RJ, Kabbur MB, Vulliet PR, Hyde DM. Substance P primes the formation of hydrogen peroxide and nitric oxide in human neutrophils. *J Leukoc Biol* 1999;65:834–840.
- Tanabe T, Otani H, Bao L, Mikami Y, Yasukura T, Ninomiya T, Ogawa R, Inagaki C. Intracellular signaling pathway of substance P-induced superoxide production in human neutrophils. *Eur J Pharmacol* 1996; 299:187–195.
- Lucattelli M, Fineschi S, Geppetti P, Gerard NP, Lungarella G. Neurokinin-1 receptor blockade and murine lung tumorigenesis. *Am J Respir Crit Care Med* 2006;174:674–683.
- Sterner-Kock A, Vesely KR, Stovall MY, Schelegle ES, Green JF, Hyde DM. Neonatal capsaicin treatment increases the severity of ozone-induced lung injury. *Am J Respir Crit Care Med* 1996;153:436–443.
- Vesely KR, Hyde DM, Stovall MY, Harkema JR, Green JF, Schelegle ES. Capsaicin-sensitive C-fiber-mediated protective responses in ozone inhalation in rats. *J Appl Physiol* 1999;86:951–962.
- Pino MV, Levin JR, Stovall MY, Hyde DM. Pulmonary inflammation and epithelial injury in response to acute ozone exposure in the rat. *Toxicol Appl Pharmacol* 1992;112:64–72.
- Pino MV, Stovall MY, Levin JR, Devlin RB, Koren HS, Hyde DM. Acute ozone-induced lung injury in neutrophil-depleted rats. *Toxicol Appl Pharmacol* 1992;114:268–276.
- Emonds-Alt X, Dautrempuich JD, Heaulme M, Neliat G, Santucci V, Steinberg R, Vilain P, Bichon D, Ducoux JP, Proietto V. *In vitro* and *in vivo* biological activities of SR140333, a potent non-peptide tachykinin NK1 receptor antagonist. *Eur J Pharmacol* 1993;250:403–413.
- Schelegle ES, Alfaro MF, Putney L, Stovall M, Tyler N, Hyde DM. Effect of C-fiber-mediated, ozone-induced rapid shallow breathing on airway epithelial injury in rats. *J Appl Physiol* 2001;91:1611–1618.
- Alfaro MF, Putney L, Tarkington BK, Hatch GE, Hyde DM, Schelegle ES. Effect of rapid shallow breathing on the distribution of ¹⁸O-labeled ozone reaction product in the respiratory tract of the rat. *Inhal Toxicol* 2004;16:1–9.
- Castleman, WL, Tyler WS, Dungworth DL. Lesions in respiratory bronchioles and conducting airways of monkeys exposed to ambient levels of ozone. *Exp Mol Pathol* 1977;26:384–400.
- Schelegle ES, Walby WF, Alfaro MF, Wong VJ, Putney L, Stovall MY, Sterner-Kock A, Hyde DM, Plopper CG. Repeated episodes of ozone inhalation attenuates airway injury/repair and release of substance P, but not adaptation. *Toxicol Appl Pharmacol* 2003;186:127–142.
- Yu Z, Cheng G, Huang X, Li K, Cao X. Neurokinin-1 receptor antagonist SR140333: a novel type of drug to treat cerebral ischemia. *Neuroreport* 1997;8:2117–2119.
- Di Sebastiano P, Grossi L, Di Mola FF, Angelucci D, Friess H, Marzio L, Innocenti P, Buchler MW Sr. 140333, a substance P receptor antagonist, influences morphological and motor changes in rat experimental colitis. *Dig Dis Sci* 1999;44:439–444.
- Castro-Obrégón S, Rao RV, del Rio G, Chen SF, Poksay KS, Rabizadeh S, Vesce S, Zhang XK, Swanson RA, Bredesen DE. Alternative, nonapoptotic programmed cell death: mediation by arrestin 2, ERK2, and Nur77. *J Biol Chem* 2004;279:17543–17553.
- Schuilting M, Zuidhof AB, Zaagsma J, Meurs H. Involvement of tachykinin NK-1 receptor in the development of allergen-induced airway hyperreactivity and airway inflammation in conscious, unrestrained guinea pigs. *Am J Respir Crit Care Med* 1999;159:423–430.
- Koren HS, Devlin RB, Graham DE, Mann R, McGee MP, Horstman DH, Kozumbo WJ, Becker S, House DE, McDonnell WF. Ozone-induced inflammation in the lower airways of human subjects. *Am Rev Respir Dis* 1989;139:407–415.
- Park J-W, Taube C, Joetham A, Takeda K, Kodama T, Dakhama A, McConville G, Allen CB, Sfyroera G, Shultz LD, et al. Complement activation is critical to airway hyperresponsiveness after acute ozone exposure. *Am J Respir Crit Care Med* 2004;169:726–732.
- Van der Kleij HP, Kraneveld AD, Redegeld FA, Gerard NP, Morteau O, Nijkamp FP. The tachykinin NK1 receptor is crucial for the development of airway inflammation and hyperresponsiveness. *Eur J Pharmacol* 2003;476:249–255.

42. Zhang WP, Zhao MH, Tian J, Yu YP, Chen JS, Wei EQ. Effect of SR-140333, a tachykinin NK-1 antagonist, on antigen-induced airway hyperresponsiveness in sensitized rats. *Yao Xue Xue Bao* 1997;32:569-572. [in Chinese]
43. Sertl K, Wiedermann CJ, Kowalski ML, Hurtado S, Plutchok J, Linnoila I, Pert CB, Kaliner MA. Substance P: the relationship between receptor distribution in rat lung and the capacity of substance P to stimulate vascular permeability. *Am Rev Respir Dis* 1988;138:151-159.
44. Nakamura M, Ofuji K, Chikama T, Nishida T. Combined effects of substance P and insulin-like growth factor-1 on corneal epithelial wound closure of rabbit *in vivo*. *Curr Eye Res* 1997;16:275-278.
45. Kuo H-P, Lin H-C, Hwang K-H, Wang C-H, Lu L-C. Lipopolysaccharide enhances substance P-mediated neutrophil adherence to epithelial cells and cytokine release. *Am J Respir Crit Care Med* 2000;162:1891-1897.
46. Bhatia M, Saluja AK, Hofbauer B, Frossard J-L, Lee HS, Castagliuolo I, Wang C-C, Gerard N, Pothoulakis C, Steer ML. Role of substance P and the neurokinin 1 receptor in acute pancreatitis and pancreatitis-associated lung injury. *Proc Natl Acad Sci USA* 1998;95:4760-4765.
47. Cao T, Pinter E, Al-Rashed S, Gerard N, Hoult JR, Brain SD. Neurokinin-1 receptor agonists are involved in mediating neutrophil accumulation in the inflamed, but not normal, cutaneous microvasculature: an *in vivo* study using neurokinin-1 receptor knockout mice. *J Immunol* 2000;164:5424-5429.
48. Pinter E, Brown B, Robin J, Hoult S, Brian SD. Lack of evidence for tachykinin NK1 receptor-mediated neutrophil accumulation in the rat cutaneous microvasculature by thermal injury. *Eur J Pharmacol* 1999;369:91-98.
49. Roch-Aweiller M, Regoli D, Chanaud B, Lenoir M, Muntaner O, Stralzko S, Giroud J-P. Tachykinins: effects on motility and metabolism of rat polymorphonuclear leucocytes. *Pharmacology* 1986;33:266-273.