 Substance P acts via the neurokinin receptor 1 to elicit bronchoconstriction, oxidative stress, and upregulated ICAM-1 expression after oil smoke exposure

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Li P-C, Chen W-C, Chang L-C, Lin S-C. Substance P acts via the neurokinin receptor 1 to elicit bronchoconstriction, oxidative stress, and upregulated ICAM-1 expression after oil smoke exposure. Am J Physiol Lung Cell Mol Physiol 294: L912–L920, 2008. First published March 7, 2008; doi:10.1152/ajplung.00443.2007.—This study aimed to 1) assess whether substance P (SP) acts via neurokinin (NK)-1 and NK-2 receptors to stimulate neurogenic inflammation (indicated by formation of ICAM-1 expression and oxidative stress) following oil smoke exposure (OSE) in rats; and 2) determine if pretreatment with antioxidants ameliorates the deleterious effects of OSE. Rats were pretreated with NK-1 receptor antagonist CP-96345, NK-2 receptor antagonist SR-48968, vitamin C, or catechins. OSE was for 30–120 min. Rats were killed 0–8 h later. Total lung resistance (Rt), airway smooth muscle activity (ASMA), lung ICAM-1 expression, neurogenic plasma extravasation (via India ink and Evans blue dye), bronchoalveolar lavage fluid SP concentrations, and reactive oxygen species formation [via lucigenin- and luminally-amplified chemiluminescence (CL)] were assessed. Lung histology was performed. SP concentrations increased significantly in nonpretreated rats following OSE in a dose-dependent manner. Rt and total ASMA increased over time after OSE. Vitamin C and catechin pretreatments were associated with significantly reduced lucigenin CL 2 and 4 h after OSE. Pretreatment with catechins significantly reduced luminal CL counts 4 and 8 h after OSE. Evans blue levels were significantly reduced following 60 and 120 min of OSE in catechin- and CP-96345-pretreated rats. ICAM-1 protein expression was significantly decreased in all pretreatment groups after OSE. Thickening of the alveolar capillary membrane, focal hemorrhaging, interstitial pneumonitis, and peribronchial inflammation were apparent in OSE lungs. These findings suggest that SP acts via the NK-1 receptor to provoke neurogenic inflammation, oxidative stress, and ICAM-1 expression after OSE in rats.

reactive oxygen species; intracellular adhesion molecule-1; acute lung injury

ACTIVATION OF NONMYELINATED bronchopulmonary C-fiber endings by various stimuli [including cigarette smoke (9), airway surface osmolality changes (14), and fire smoke (22)] causes the release of tachykinins and calcitonin gene-related peptide neuropeptides stored in the peripheral nerve terminals, eliciting both central nervous system (CNS) and local axon reflex responses (4). The CNS reflex consists of bronchoconstriction, inspiratory apnea, rapid shallow breathing, cough, hypotension, and bradycardia, whereas the axon reflex response initiates local inflammation, increased mucous secretion, and bronchoconstriction. Substance P (SP), a tachykinin neuropeptide, induces a range of neuroimmunomodulatory effects on target smooth muscle or parasympathetic ganglia cells expressing neurokinin (NK)-1 or NK-2 receptors (1, 2). These effects, collectively termed neurogenic airway inflammation, comprise bronchoconstriction, microvascular plasma leakage, neutrophil recruitment, and inflammatory mediator synthesis. SP-mediated neutrophil adhesion to bronchial epithelial and endothelial cells further augments the proinflammatory response and oxidative stress via production of ICAM-1, chemokines/cytokines, arachidonic acid products, and reactive oxygen species (ROS)/nitric oxide (NO) derivatives such as hydroxyl radicals and superoxides (2, 3, 8).

Exposure to mutagenic and genotoxic pollutants of oil fume emitted during pyrolysis degrades epithelial integrity and is associated with the development of lung and bladder cancer (6). Previous studies have indicated that there are 12 major mutagenic aldehyde compounds in peanut sunflower and lard oil (23–25). The most potent of these, trans,trans-2,4-decadienal, has been demonstrated to cause significant ROS-related oxidative damage through 8-hydroxy-2-deoxyguanosine formation in A-549 lung adenocarcinoma cells (23). Significant increases in bronchoalveolar lavage fluid (BALF) SP concentrations have been reported in rabbits following smoke exposure (17). Substantial elevations in TNF-α gene and protein expression, superoxide anion levels, epithelial cell necrosis, and alveolar hypoventilation were also noted (21). In a more recent study, we (10) demonstrated that electrical stimulation of SP containing afferent thoracic vagus nerves (TVN) resulted in upregulation of NF-κB expression, plasma extravasation, and ICAM-1 in the rat respiratory tract. SP release increased with the frequency of TVN stimulation as did the severity of microvascular permeability, inflammatory cell infiltration, and oxidative stress.

Numerous animal and human studies have suggested that various mechanisms underlie airway hyperresponsiveness following hypoxia and exposure to oil or fire smoke. Experimental trials have characterized the cytotoxic and genotoxic effects of methanolic extracts of various oil fume variants (sunflower, soybean, and lard) on human lung carcinoma cells with respect to ROS/NO formation (5, 10, 24). However, the precise role that SP plays in mediating neurogenic inflammation through interaction with NK-1 receptor and NK-2 receptor and consequent ROS and ICAM-1 formation following oil smoke exposure (OSE) is unclear.

The objective of this study was to assess the role that SP plays in mediating the neurogenic inflammation associated with exposure to smoke from lard oil. Specifically, we wished to examine the importance of the interaction of SP with NK-1 and NK-2 receptors and consequent changes ICAM-1 expres-
sion and oxidative stress. We hypothesized that OSE would: 1) enhance the tachykininergic and cholinergic mechanisms involved in bronchoconstriction; and 2) cause the release of SP, which would then interact with NK-1 or NK-2 receptors and initiate airway hyperactivity through increased ICAM-1 expression and ROS formation. Thus we further hypothesized that pretreatment with an NK receptor antagonist or antioxidant supplement would reduce/ameliorate the neurogenic inflammation and oxidative injury associated with OSE.

METHODS

Animals and drug preparation. Male Sprague-Dawley rats (weighing between 200–250 g and aged 12–14 wk) were housed in temperature-controlled facilities at the Experimental Animal Center, I-Shou University, where the light cycle was from 0700 to 1800. Animal care and experimental protocols were in accordance with guidelines prescribed by the National Science Council of the Republic of China (NSC1997). The animal studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of I-Shou University, Kaohsiung, Taiwan (IACUC Protocol no. AUP-95-55-001 and Approval no. IACUC-ISU-95004).

Rats were anesthetized with subcutaneous urethane (1.2 g/kg; Sigma-Aldrich, St. Louis, MO). Femoral vein catheters (PE-50; Clay Adams, Parsippany, NJ) were inserted to facilitate blood sampling and drug administration. Blood vessel patency was maintained by intravenous infusion of saline at a rate of 1.2 ml/min via an infusion pump (Infors, Bottmingen, Switzerland). Femoral arterial catheters were placed for measurement of arterial blood pressure. This was recorded using a polygraph (ML845 PowerLab 4/25 T system; ADInstruments, Sydney, Australia).

On the experimental day, groups of rats were pretreated with either a NK-1 receptor antagonist (CP-96345; Sanofi Recherche, Montpellier, France), NK-2 receptor antagonist (SR-48968, Sanofi Recherche), H$_2$O$_2$ scavenger (vitamin C; Sigma Chemical, St. Louis, MO), or an O$_2$ and H$_2$O$_2$ scavenger (catechins). CP-96345 and SR-48968 were dissolved in 10% dimethyl sulphoxide in 0.9% saline at concentrations of 1 µg/kg. Vitamin C (1 mg/ml) and catechins (328 mg/g epigallocatechin gallate, 132 mg/g epicatechin, 108 mg/g epigallocatechin, 104 mg/g galloatechin, and 44 mg/g catechin; Numan Biotech, Taipei, Taiwan) were dissolved in 0.9% saline at a concentration of 2.5 mg/kg. All drugs were administered as a bolus via the femoral catheter. Atropine (1 mg·ml$^{-1}$·kg$^{-1}$) was given 20 min before OSE to inhibit cholinergic innervation of the airway nervous system.

OSE. Ten minutes after pretreatment, rats were placed in an experimental chamber and exposed to oil smoke for 30, 60, 90, or 120 min via a custom-made inhalation system and artificial respirator. Control rats were also placed in the chamber but were exposed to ambient room air only. To produce the smoke, 500 ml of lard was placed in a ceramic crucible and burned in a stainless steel smoke chamber. The thermal furnace system produced a consistent high temperature of 350°C. Generated effluent oil smoke was vacuum-transferred through an inverted funnel canopy into a sealed chamber at a continuous flow (dynamic) rate of 3 l/min. The mean smoke temperature for all exposures was 32.6 ± 1.7°C.

Oil smoke was analyzed for O$_2$ (Model 2200) oxygen analyzer; California Analytical Instruments, Orange, CA), CO$_2$ (Model 2010 NDIR analyzer, California Analytical Instruments), nitrite and nitrate (NO$_2$; Model 2020 NO$_X$ analyzer, California Analytical Instruments), and particulate (CIS 100 analyzer, California Analytical Instruments) content. We found that oil smoke generated in this manner contains ~2.8% O$_2$, 18% CO$_2$, 34% CO, and 86 mg/l particulates.

Rats were killed immediately and up to 8 h following OSE to assess lung injury (specific details of this follow). A further subset of (nonpretreated) rats were exposed to OSE for 0–120 min and killed thereafter between 0–8 h for assessment of SP and ROS in BALF.

Measurement of total lung resistance and airway smooth muscle activity. Total lung resistance ($R_{L}$) and airway smooth muscle activity (ASMA) were measured in all rats to serve as indices of the NK receptor-mediated bronchoconstrictive response. Briefly, trachea was cannulated (PE-200, Clay Adams) caudal to the larynx, and animals breathed spontaneously through a pneumotachometer (TSD 137C; Biopac Systems, Goleta, CA) connected to a flow transducer (TSD 160A, Biopac Systems) to monitor airflow with a zero-flow method. Intratracheal pressure was measured using a pressure transducer (Model DP 103-24; Validyne, Northridge, CA) connected to a manometer (Model CD-15-A-1-B-1, Validyne).

A fluid-filled PE-50 cannula was introduced into the esophagus to measure esophageal pressure to give an indication of pleural pressure. Transpulmonary pressure (defined as the pressure difference between the intratracheal and the esophageal pressures) was measured with a manometer. $R_{L}$ was calculated as previously described (10).

For electromyogram (EMG) recordings of ASMA, epoxy-coated stainless steel wire (50 µm; M.T. Giken, Tokyo, Japan) was placed in the inner layer of airway smooth muscle of the lower trachea using a dissecting microscope. The EMG electrodes were embedded at a depth of 1–2 mm in smooth muscle. EMG signals were amplified and recorded on a recording system (ML845 PowerLab 4/25 T system; ADInstruments). All data were stored on a computer and analyzed using PowerLab Chart Pro software (ADInstruments).

BALF. At the designated time points following OSE, rats were anesthetized to surgical depth with urethane (0.75 mg/kg ip ethyl carbamate). The trachea was exposed and intubated using an 18-gauge needle covered with polyethylene tubing (2.29-mm outer diameter). Following intubation, rats were given an overdose of intravenous sodium pentobarbital (120 mg/kg). Using a 10-ml syringe, 7 ml of PBS (pH 7.4) was then instilled into the lungs. Lungs were washed five times before lavage fluid (~6 ml) was collected. Cells were isolated from the BALF by centrifugation at 2,500 rpm for 10 min. The resultant supernatant was stored at −80°C for later assessment of cytokine levels, and the cells were resuspended in 1.0-ml sterile isotonic saline.

Commercially available ELISA kits (R&D Systems, Minneapolis, MN) were used to determine IL-1β and TNF-α levels.

SP enzyme immunoassay. SP levels in BALF supernatant were quantified using a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) following the manufacturer’s instructions. Concentrations of SP in BALF are expressed in picograms per milliliter.

ROS in BALF: lucigenin- and luminol-amplified chemiluminescence determination. This methodology has been described in detail previously (10).

Morphometric analysis of India ink-labeled leaky blood vessels and measurement of Evans blue dye. To evaluate neurogenic plasma extravasation, rats were given intravenous injections of India ink (1 mg/kg; Chroma-Gesellschaft, Königen, Germany) over 5 s or Evans blue dye (50 mg/kg) following OSE. Animals were killed following infusion of 50 ml of 90% (wt/vol) saline (37°C) into the left cardiac ventricle. The two tracheal halves and mainstem bronchi, left cranial lateral and medial caudal lobar bronchi, and the right cranial (1st), medioventral (2nd), caudal (3rd), and accessory (4th) lobar bronchi were examined. India ink was detected and quantified using a microscope (Leica DMRB; Leica, Wetzlar, Germany) as previously described (10). Evans blue dye was detected by measuring absorbance on a spectrophotometer (Beckman Coulter DU 6408) at 620 nm after extraction of a known volume of formamide at 60°C for 24 h.

Concentrations of Evans blue dye in micrograms per gram were estimated from a calibration curve constructed using 12 standard dye solutions. The average Evans blue concentration in the left secondary bronchi was calculated from the Evans blue concentration and the weight (W) of the left cranial (LCr) and caudal (LCa) lobar bronchi using the equation $[(LCr\times W_{LCr})+(LCa\times W_{LCa})]/(W_{LCr}+W_{LCa})$. The average Evans blue concentration for the right secondary bronchi was calculated from the Evans blue concentration and the weight (W) of the right cranial (RCr) and caudal (RCa) lobar bronchi using the equation $[(RCr\times W_{RCr})+(RCA\times W_{RCA})]/(W_{RCr}+W_{RCA})$.
bronchi was calculated from the Evans blue concentration and the weight (W) of the right cranial (RCr), medius (RMe), caudal (RCa), and accessory (RAc) lobar bronchi using the equation:

\[
\frac{(RCr/H11003 WRCr)}{(RMe/H11003 WRMe)} \frac{(RCa/H11003 WRCa)}{(RAc/H11003 WRAc)}
\]

Histological and immunohistochemical analyses. At various times (previously designated) following OSE stimulation, lungs were removed and fixed by perfusing 10% buffered formalin through the trachea at a pressure of 30 cmH2O for 24 h. Lungs were then dehydrated through a series of ethanol solutions, cleared with methyl salicylate, and embedded in paraffin. Sections of the lung tissue (5 μm) were mounted on glass slides and stained with hematoxylin and eosin for examination by light microscopy. For immunohistochemical analysis, the sections were first treated with blocking buffer for 15 min to block nonspecific background binding. Primary rabbit antiserum (1:1,000 anti-ICAM-1) was then applied overnight at 4°C. After rinses in PBS, sections were treated with the Zymed Histostain kit (Zymed Laboratories, South San Francisco, CA). This included secondary biotinylated goat anti-rabbit serum followed by horseradish peroxidase-labeled streptavidin and subsequent development in a diaminobenzidine substrate solution, yielding a permanent reddish brown reaction product. All slides were coverslipped permanently with crystal mount.

Immunoblot analysis for heat shock protein 70 and ICAM-1. Heat shock protein 70 (Hsp 70) and ICAM-1 in right lung were assessed after OSE. For protein analysis, lung samples were homogenized with a prechilled mortar and pestle in extraction buffer (10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 1 mM PMSF, 1% Nonidet P-40, 0.5% deoxycholate, 2% 2-mercaptoethanol, 10 μg/ml pepstatin A, and 10 μg/ml aprotinin). The homogenate was maintained at 4°C for 30 min and then centrifuged at 12,000 g for 12 min at 4°C, and the resultant supernatant was collected. Protein concentrations were determined by Bio-Rad protein assay.

SDS-PAGE was performed using 12.5% separation gels in the absence of urea and stained with Coomassie brilliant blue. Proteins on the SDS-PAGE gels (each lane contained 30 μg of total protein) were transferred to nitrocellulose filters. Immunoreactive bands were detected by incubating with primary antibodies for ICAM-1 (R&D Systems) and Hsp 70 (R&D Systems) for ~4 h followed by secondary antibody alkaline phosphatase (1 h) and finally nitro blue tetrazolium and a 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Roche Diagnostics, Mannheim, Germany) stock solution for 30 min at room temperature.

Statistical analyses. Normally distributed continuous variables were compared by one-way ANOVA. When a significant difference between groups was apparent, multiple comparisons of means were performed using the Bonferroni procedure with type I error adjust-

![Fig. 1. Time course of substance P bronchoalveolar lavage fluid concentrations following oil smoke exposure (OSE) for 0, 30, 60, or 120 min. a: A statistically significant difference between the indicated treatment group and the control group (i.e., no OSE) at a given time after OSE. b: A statistically significant difference between the indicated treatment group and the OSE 30-min group at a given time after OSE. c: A statistically significant difference between the OSE 60-min and OSE 120-min group at a given time after OSE. Pairwise multiple comparisons between groups were determined using Bonferroni test with α = 0.008 adjustment.](http://ajplung.physiology.org/)

![Fig. 2. Effect of pretreatment on pulmonary resistance (Rl), airway smooth muscle electromyogram (EMG) activity (B), and blood pressure (BP, C) following 0, 30, 60, or 120 min of OSE. Rats were pretreated with CP-96345, SR-48968, vitamin C, or catechins. a: A statistically significant difference between the indicated treatment group and the control group (i.e., no pretreatment) at a given time after OSE. b: A statistically significance difference between the indicated treatment group and the OSE (i.e., OSE pretreatment for 120 min) at a given time after OSE. *A statistically significant difference between the given group and its corresponding baseline group (i.e., 0 OSE). Pairwise multiple comparisons between groups were determined using Bonferroni test with α = 0.017 adjustment.](http://ajplung.physiology.org/)
ment. Data are presented as means ± SE. All statistical assessments were two-sided and evaluated at the 0.05 level of significance. Statistical analyses were performed using SPSS 15.0 statistics software (SPSS, Chicago, IL).

RESULTS

SP concentrations in BALF following OSE. Concentrations of SP in BALF were significantly increased in all groups following OSE between 0–4 h (Fig. 1). There was a dose-response effect evident where SP concentrations were highest in OSE 120 (OSE for 120 min) rats, intermediate in OSE 60 rats, and lowest in OSE 30 rats. Maximum SP levels were apparent 4 h after OSE in rats exposed for 60 and 120 min (9.28 ± 0.86 and 15.09 ± 1.33 pg/ml, respectively), whereas the peak level in the OSE 30 group was 5.38 ± 0.70 pg/ml 120 min after the intervention. There were gradual SP concentration reduction in OSE 120 and OSE 160 rats between 4–8 h. SP levels in OSE 30 rats did not change markedly between 4–8 h but were still significantly lower than those in both other groups at 8 h.

Pulmonary function (RL), ASMA, and blood pressure following OSE. OSE (from 0 to 120 min) increased total RL and ASMA activity in a time-dependent manner in all groups (Fig. 2, A and B). Statistically significant increases in RL were

![Histopathological slides from lung tissue following 120-min OSE.](http://ajplung.physiology.org/)

Fig. 3. Representative histopathological slides from lung tissue following 120-min OSE. A: section from a control lung. (B and C). Section from a rat exposed to OSE indicating interstitial pneumonitis (i) and thickening of the alveolar-capillary membrane (a). D: section from a rat exposed to OSE revealing sloughing and necrosis of bronchiolar epithelium (e), neutrophils (thin arrows), peribronchiolar inflammation (thick arrows), and interstitial pneumonitis (i). E and F: section demonstrating an increased number of leukocytes (l; red arrows) and neutrophils (n; black arrows) following OSE. G and H: sections from a rat pretreated with catechins before exposure to OSE demonstrating decreased alveolar-capillary membrane (a) thickness, markedly reduced interstitial pneumonitis (i), fewer neutrophils, intact bronchiolar epithelium (e), and only scattered leukocytes (red arrows) in the peribronchiolar space. Magnification was ×200 for A–D and G and H and ×400 for E and F.
Table 1. Lucigenin- and luminal-enhanced CL in BALF after OSE

<table>
<thead>
<tr>
<th>Sample CL, counts per 10 s</th>
<th>OSE 0 (n = 5)</th>
<th>OSE 30 (n = 5)</th>
<th>OSE 60 (n = 5)</th>
<th>OSE 120 (n = 5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lucigenin CL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>74.60±110.08</td>
<td>86.80±70.79</td>
<td>97.80±80.33</td>
<td>88.60±20.69</td>
<td>0.279</td>
</tr>
<tr>
<td>1 h after OSE</td>
<td>78.60±100.54</td>
<td>232.00±230.74</td>
<td>339.00±330.10</td>
<td>594.20±370.12</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>2 h after OSE</td>
<td>89.20±90.98</td>
<td>416.20±330.85</td>
<td>687.20±530.01</td>
<td>1,021.00±770.42</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>4 h after OSE</td>
<td>98.80±90.77</td>
<td>821.20±390.92</td>
<td>1,059.40±680.73</td>
<td>1,616.20±1,680.86</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>8 h after OSE</td>
<td>92.60±80.94</td>
<td>704.00±600.32</td>
<td>853.60±590.45</td>
<td>1,097.20±1,050.40</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td><strong>Luminal CL</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>80.80±60.37</td>
<td>81.40±50.37</td>
<td>77.20±50.54</td>
<td>75.80±60.49</td>
<td>0.889</td>
</tr>
<tr>
<td>1 h after OSE</td>
<td>77.20±70.32</td>
<td>223.60±290.60</td>
<td>304.40±310.38</td>
<td>427.20±600.00</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>2 h after OSE</td>
<td>89.00±40.30</td>
<td>312.00±320.04</td>
<td>397.00±450.21</td>
<td>571.40±410.79</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>4 h after OSE</td>
<td>83.60±80.35</td>
<td>717.80±820.84</td>
<td>996.60±660.87</td>
<td>1,021.00±1,050.95</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>8 h after OSE</td>
<td>84.80±70.71</td>
<td>562.00±510.95</td>
<td>508.80±500.00</td>
<td>934.00±560.75</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

P values are based on ANOVA test; *P < 0.05. †Statistically significant difference between the indicated treatment group and the control group (i.e., no oil smoke exposure (OSE)) at a given time after OSE. §Statistically significant difference between the indicated treatment group and the OSE 30-min group at a given time after OSE. *Statistically significant difference between the OSE 60- and 120-min groups at a given time after OSE. §Statistically significant difference between the given group and its corresponding baseline group (i.e., 0 OSE). Pairwise multiple comparisons between groups were determined using Bonferroni test with α = 0.008 (†, ¶, and †) and 0.017 (§) adjustment. CL, chemiluminescence; BALF, bronchoalveolar lavage fluid.

Table 2. Lucigenin- and luminal-enhanced CL in BALF at various times for the various pretreatment groups following 120 min of OSE

<table>
<thead>
<tr>
<th>Sample CL, counts per 10 s</th>
<th>OSE 120 (n = 5)</th>
<th>CP-96345 + OSE (n = 5)</th>
<th>SR-48968 + OSE (n = 5)</th>
<th>Vitamin C + OSE (n = 5)</th>
<th>Catechins + OSE (n = 5)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lucigenin CL</strong></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>88.60±20.69</td>
<td>81.20±50.30</td>
<td>73.60±40.47</td>
<td>95.40±60.01</td>
<td>81.00±50.24</td>
<td>0.048*</td>
</tr>
<tr>
<td>1 h after OSE</td>
<td>594.20±370.12</td>
<td>446.80±360.91†</td>
<td>525.60±680.58‡</td>
<td>354.80±440.44‡</td>
<td>379.80±450.69‡</td>
<td>0.011*</td>
</tr>
<tr>
<td>2 h after OSE</td>
<td>1,021.00±770.42</td>
<td>818.80±430.40‡</td>
<td>827.00±550.93‡</td>
<td>566.80±530.23‡</td>
<td>488.20±450.30‡</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>4 h after OSE</td>
<td>1,616.20±1,680.86</td>
<td>1,070.40±880.55‡</td>
<td>1,102.80±1,080.50‡</td>
<td>872.20±490.22‡</td>
<td>774.20±600.17‡</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>8 h after OSE</td>
<td>1,097.20±1,060.12</td>
<td>919.20±620.88‡</td>
<td>981.80±1,060.12‡</td>
<td>720.20±440.49‡</td>
<td>693.20±580.49‡</td>
<td>0.008*</td>
</tr>
<tr>
<td><strong>Luminal CL</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>75.80±60.49</td>
<td>80.40±50.68</td>
<td>77.60±70.65</td>
<td>75.80±70.26</td>
<td>85.00±40.45</td>
<td>0.829</td>
</tr>
<tr>
<td>1 h after OSE</td>
<td>427.20±600.38</td>
<td>406.20±500.38</td>
<td>422.20±460.79</td>
<td>375.80±390.02†</td>
<td>284.00±330.15</td>
<td>0.217</td>
</tr>
<tr>
<td>2 h after OSE</td>
<td>571.40±410.79‡</td>
<td>513.80±410.93</td>
<td>538.40±260.69</td>
<td>461.00±310.09</td>
<td>402.60±340.38‡</td>
<td>0.026*</td>
</tr>
<tr>
<td>4 h after OSE</td>
<td>1,021.00±1,050.95</td>
<td>725.40±880.73</td>
<td>894.00±490.06</td>
<td>670.00±460.75</td>
<td>573.40±420.53‡</td>
<td>0.001*</td>
</tr>
<tr>
<td>8 h after OSE</td>
<td>934.00±560.75‡</td>
<td>759.60±650.44</td>
<td>876.40±500.74</td>
<td>674.60±450.35</td>
<td>607.40±610.06‡</td>
<td>0.003*</td>
</tr>
</tbody>
</table>

P values are based on ANOVA test; *P < 0.05. †Statistically significant difference between the indicated treatment group and the control group (i.e., no oil smoke exposure (OSE)) at a given time after OSE. §Statistically significant within group difference between the given time point and the control time point. Pairwise multiple comparisons between groups were determined using Bonferroni test with α = 0.0125 adjustment.
There were no significant differences in luminal CL counts at 0 and 1 h after OSE between the five groups. Pretreatment with vitamin C and catechins significantly reduced lucigenin CL counts by 19% and 30%, respectively, 2 h after OSE, 34% and 47%, respectively, 4 h after OSE, and 28% and 35%, respectively, 8 h after OSE.

Effects of NK antagonists and antioxidants on OSE-induced plasma extravasation. India ink and Evans blue measures increased in proportion to the duration of OSE in all groups. There were no significant baseline differences between the groups for either measure (Table 3). Pretreatment with vitamin C and catechins significantly reduced India ink concentrations by 26% and 40%, respectively, in the OSE 60 group. CP-96345 pretreatment reduced India ink concentrations by 46% in this group. In the OSE 120 group, pretreatment with vitamin C, catechins, and CP-96345 significantly reduced India ink concentrations by 27%, 42%, and 46%, respectively. SR-48968 resulted in slight but significant reductions in India ink concentrations.

Pretreatment with vitamin C and catechins significantly reduced Evans blue concentrations by 37% and 54%, respectively, after 60 min of OSE and by 29% and 41%, respectively, after 120 min of OSE compared with corresponding concentrations in nonpretreated rats. CP-96345 pretreatment resulted in significantly reduced (44%) Evans blue levels following 60 min of OSE and reduced 35% following 120 min of OSE. SR-48968 pretreatment resulted in a slight but significant reduction in India ink concentrations.

Effect of NK antagonists and antioxidants on OSE-induced ICAM-1 and Hsp expression in the lung.

![Representative Western blots](http://ajplung.physiology.org)
ing OSE and the various pretreatments are presented in Fig. 4. ICAM-1 expression was significantly upregulated in the non-pretreated group following 30–120 min of OSE. Compared with the nonpretreated group after 120 min of OSE, expression levels of ICAM-1 were lower in all pretreatment groups (Fig. 4, A and B). β-Actin expression remained constant.

In nonpretreated rats, Hsp 70 expression was undetectable following 30 min of OSE (Fig. 4, C and D). Hsp 70 was detected at 60 min following OSE and further increased following 120-min OSE. Expression of lung Hsp 70 following 120 min of OSE from CP-96345- and SR-48968-pretreated rats appeared similar to that in lungs from nonpretreated rats. Expression of Hsp 70 was higher in lungs from vitamin C- and catechin-pretreated rats (Fig. 4, C and D).

The immunohistological findings regarding ICAM-1 cross-linking with local lung vascular endothelium and inflammatory cells are presented in Fig. 5.

Figure 6 shows TNF-α and IL-1β concentrations following OSE. TNF-α levels were significantly higher in each OSE group compared with the control group (no OSE; \(P < 0.017\); Fig. 6A). In contrast, IL-1β concentrations were only significantly higher in the OSE 120 compared with the control group (\(P < 0.001\); Fig. 6B). Pretreatment with vitamin C and catechins before OSE resulted in significantly lower TNF-α levels compared with the OSE-only group (decreases were 48% and 43%, respectively; Fig. 6C). Pretreatment with CP-96345, vitamin C, and catechins was associated with significantly decreased IL-1β concentrations compared with the OSE-only group (Fig. 6D).

**DISCUSSION**

The major objectives of this study were to determine whether SP acts via the NK-1 or NK-2 receptor to elicit airway hyperactivity following OSE in rats and to also determine whether injury associated with the formation of oxygen free radical could be ameliorated by pretreatment with antioxidants. Our findings support the notion that airway hyperactivity associated with OSE is at least in part due to SP interacting with the NK-1 receptor and indicate that pretreatment with antioxidants leads to decreased ROS formation and neurogenic inflammation.

At 300°C, the average yield of total aliphatic aldehydes is less than 0.5 mm·mg\(^{-1}\)·min\(^{-1}\) from most types of oil (5, 12). The average yield of total aldehydes from soybean and lard oil at 150°C, however, is ~1.5 ppm·mg\(^{-1}\)·min\(^{-1}\), indicating that components of lard are pyrolyzed at lower temperatures (12). Pork lard at temperatures of 350–400°C produces the highest amount of formaldehyde (10.9 mm·mg\(^{-1}\)·min\(^{-1}\)), acetaldehyde (1.04 ppm·mg\(^{-1}\)·min\(^{-1}\)), and acrolein (0.66 ppm·mg\(^{-1}\)·min\(^{-1}\)) via thermolytic decomposition and oxygenation of saturated fatty acids (5). These hydroxalkenal compounds of lipid peroxidation lead to decreased antioxidant enzyme activity, in particular that of glutathione (GSH); an antioxidant enzyme that mediates cellular metabolism and homeostasis against free radical damage through the GSH redox cycle (Ref. 17). The activities of S-transferase and GSH reductase are inhibited as a result of cellular oxidation caused by oil fumes in cooking oil. This leads to microvascular leakage, cell death, and DNA damage (24). Increased formation of ROS in addition to the release of various cytokines, ICAM-1, and arachidonic acid production associated with the SP-mediated proinflammatory response result in vascular constriction, bronchoconstriction, neurogenic inflammation, endogenous antioxidant depletion, hemodynamic derangements, and oxidative injuries (10).

Although we were not able to directly quantify ROS and NO in the present study, our indirect assessment of these variables (via measurement of lucigenin and luminal CL) indicated that levels were significantly increased within 4 h of OSE. Lucigenin has been demonstrated to react almost exclusively with ROS under optimal laboratory conditions and is commonly used to increase the sensitivity of cellular CL assays (20). Pretreatment with antioxidants (vitamin C and catechins) that inhibit lipid peroxidation and minimize oxidative injury were found to significantly reduce lucigenin CL and Evans blue level following prolonged OSE (60 and 120 min). Furthermore, we noted that pretreatment with both antioxidants, as well as NK-1/NK-2 receptor antagonists, significantly reduced tracheal EMG activity after 60 and 120 min of OSE. We suggest that upregulated ICAM-1 expression is a result of ROS-related inflammation following release of tachykinins.

![Fig. 5. Representative images from oil smoke-exposed (120 min) rat lungs demonstrating ICAM-1 expression by immunolabeling (dark brown, indicated by arrows) on lung vascular endothelial (En) cells (A and B) and inflammatory cells (A). Magnification was ×400 for both images.](http://ajplung.physiology.org/)
Histopathological examination of lung tissue from rats exposed to OSE revealed significant damage. This was manifested by thickening of the alveolar capillary membrane, interstitial pneumonitis, peribronchiolar inflammation, and sloughing and necrosis of the bronchiolar epithelium. These observations are consistent with previously published findings (1, 22).

When an organism is exposed to stress stimuli, the affected cells quickly respond by expressing heat shock proteins. An important member of the Hsp family is Hsp 70. Hsp 70 is known to play a role in modulating protein biosynthesis and ensures cell protection and survival at high temperatures, presumably by binding to ribosomal subunits (13). In the present study, we noted that Hsp 70 appeared to be overexpressed in rats pretreated with both vitamin C and catechins following OSE. Previous in vitro and in vivo studies have demonstrated that heat shock proteins offer protection from oxidative damage both in vitro and in vivo (7, 15, 16). Hence the increased expression of Hsp 70 in vitamin C- and catechin-pretreated rats may underlie the decreased oxidative stress and damage (in part caused by cytokine production) following OSE.

Several studies have demonstrated that SP is a proinflammatory agent that influences various signaling transduction pathways within a complex inflammatory network by C-fiber afferent stimulation following external stimulation (1, 4, 9, 14, 22). In vivo studies have demonstrated the efficacy of antioxidants and free radical scavengers in ameliorating increases in endogenous SP levels (which may result in increased microvascular permeability via upregulated ICAM-1 expression, edema, and severe hypoxia) (11). It would appear that ROS formation is related to the release and degradation of tachykinins. We noted that pretreatment with the NK-1 and NK-2 receptor antagonists CP-96346 and SR-48968 resulted in decreased ICAM-1 protein expression following 120 min of OSE. Furthermore, pretreatment with these antagonists was also associated with decreased indicators of both the CNS and local axon reflex responses and well as expression of factors in BALF known to be involved in neurogenic airway inflammation. These findings suggest that neurogenic inflammation associated with SP release is at least in part a result of interactions with the NK-1 and to lesser extent the NK-2 receptor.

Numerous studies have indicated that NK-1 receptor antagonism leads to inflammation via multiple inflammatory mediators (18, 19). Thus it would seem that NK-1 receptor antagonism might be useful as a primary therapeutic intervention to prevent or ameliorate the development of inflammatory and oxidative injury following OSE. Additional studies are warranted to further clarify the involvement of the NK-1 and NK-2

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Fig. 6. Cytokine levels in bronchoalveolar lavage fluid following OSE. A and B: TNF-α (A) and IL-1β (B) concentrations in rats exposed to OSE for 0, 30, 60, or 120 min. C and D: TNF-α (C) and IL-1β (D) concentrations in rats pretreated with CP-96345, SR-48968, vitamin C, or catechins and exposed to OSE for 120 min. *A statistically significance difference between the given group and the control group (i.e., no OSE). a: a statistically significance difference between the given group and the OSE group (i.e., no pretreatment but OSE for 120 min). Pairwise multiple comparisons between groups were determined using Bonferroni test with α = 0.017 (*) and 0.001 (a) adjustments.
receptors in mediating SP-induced inflammatory injury following OSE.

In conclusion, our findings indicate that SP interacts with the NK-1 to provoke neurogenic inflammation, oxidative stress, and upregulated ICAM-1 expression following OSE in rats. Short-term application of antioxidants and NK-1 receptor antagonists may ameliorate oxidative injury associated with OSE.

GRANTS
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