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The Neuropeptide Substance P Is a Critical Mediator of Burn-Induced Acute Lung Injury

Selena Wei Shan Sio,* Manoj Kumar Puthia,† Jia Lu,† Shabbir Moochhala,*† and Madhav Bhatia2*†

The classical tachykinin substance P (SP) has numerous potent neuroimmunomodulatory effects on all kinds of airway functions. Belonging to a class of neuropeptides targeting not only resident cells but also inflammatory cells, studying SP provides important information on the bidirectional linkage between how neural function affects inflammatory events and, in turn, how inflammatory responses alter neural activity. Therefore, this study aimed to investigate the effect of local burn injury on inducing distant organ pulmonary SP release and its relevance to lung injury. Our results show that burn injury in male BALB/c mice subjected to 30% total body surface area full thickness burn augments significant production of SP, preprotachykinin-A gene expression, which encodes for SP, and biological activity of SP-neurokinin-1 receptor (NK1R) signaling. Furthermore, the enhanced SP-NK1R response correlates with exacerbated lung damage after burn as evidenced by increased microvascular permeability, edema, and neutrophil accumulation. The development of heightened inflammation and lung damage was observed along with increased proinflammatory IL-1β, TNF-α, and IL-6 mRNA and protein production after injury in lung. Chemokines MIP-2 and MIP-1α were markedly increased, suggesting the active role of SP-induced chemotactants production in trafficking inflammatory cells. More importantly, administration of L703606, a specific NK1R antagonist, 1 h before burn injury significantly disrupted the SP-NK1R signaling and reversed pulmonary inflammation and injury. The present findings show for the first time the role of SP in contributing to exaggerated pulmonary inflammatory damage after burn injury via activation of NK1R signaling. The Journal of Immunology, 2008, 180: 8333–8341.

Substance P (SP) is a neuropeptide of the tachykinin family known for its proinflammatory effects on numerous airway effector cells of the innate and adaptive immune system. It is a major mediator of neuroimmunomodulatory activities and neurogenic inflammation within the central and peripheral nervous system and has been shown to influence numerous respiratory functions under normal and pathological conditions. Primarily, the synthesis and release of SP arise from the nociceptive responses of sensory primary afferent nerve fibers initiated by noxious stimuli and is widely distributed in most organs, including the lungs. Interestingly, recent evidence from rodent studies has shown SP to be stored inside and produced by important immune cells as well, such as in resident macrophages, circulating leukocytes, lymphocytes, and dendritic cells. Molecular studies have further established that the released SP molecules bind to a family of ubiquitous G protein-coupled receptors, of which the membrane-bound neurokinin-1 receptor (NK1R) has the highest affinity for SP (6–8). Activation of the receptor complex produces a variety of neuroimmune responses in mammalian airways including increased microvascular permeability and plasma extravasation, immune cell influx, increased edema, vasodilation, and glandular secretions, thereby contributing to heightened inflammation of the injured tissue site and neutrophil accumulation. The development of heightened inflammation and lung damage was observed along with increased proinflammatory IL-1β, TNF-α, and IL-6 mRNA and protein production after injury in lung. Chemokines MIP-2 and MIP-1α were markedly increased, suggesting the active role of SP-induced chemotactants production in trafficking inflammatory cells. More importantly, administration of L703606, a specific NK1R antagonist, 1 h before burn injury significantly disrupted the SP-NK1R signaling and reversed pulmonary inflammation and injury. The present findings show for the first time the role of SP in contributing to exaggerated pulmonary inflammatory damage after burn injury via activation of NK1R signaling.
puncture-induced, sepsis-associated lung injury (17, 18). However, none has investigated the neurogenic inflammatory effects of neuropeptides, such as SP, in mediating distant organ lung inflammation and damage after local burn injury.

In the present study, we show that SP is a key mediator in the pathogenesis of lung inflammation and damage in a burn injury model via activation of NK1R signaling and that the effect of SP-NK1R signaling on effector pulmonary cells further instigates transcriptional expression and production of proinflammatory cytokines and chemokines. Additionally, we demonstrate that SP modulates circulating levels of immune cells including neutrophils, monocytes, lymphocytes, and platelets via NK1R signaling after burn injury.

Materials and Methods

Mouse burn injury model

All experiments were approved by Institutional Animal Care and Use Committee of DSO National Laboratories and were conducted in accordance with their established guidelines. Groups of male BALB/c mice, 6 – 8 wk old, were anesthetized with ketamine (160 mg/kg) plus xylazine (4 mg/kg) and the dorsal hair was clipped. Mice were placed in an insulating mold device with an opening calculated to expose 30% total body surface area. The exposed skin was immersed in 95°C water for 8 s. This has been shown to produce an anesthetic full thickness burn (19). Sham mice,

Table 1. PCR primer sequences, optimal conditions, and product sizes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Optimal Conditions</th>
<th>Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>r18S</td>
<td>Sense: 5’-GTAACCGATTGACCCCATTT-3’</td>
<td>28 cycles</td>
<td>150</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-CCATCCAGTGGATGACCG-3’</td>
<td>Annealing: 59°C</td>
<td></td>
</tr>
<tr>
<td>NK1R</td>
<td>Sense: 5’-CTTGGTCTTTGGGAACCCGTGTG-3’</td>
<td>38 cycles</td>
<td>501</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-AGGGAGAAGCAGGGACAC-3’</td>
<td>Annealing: 59°C</td>
<td></td>
</tr>
<tr>
<td>PPT-A</td>
<td>Sense: 5’-GCAAATGAGGAAATCAGAA-3’</td>
<td>38 cycles</td>
<td>282</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-GCTTGAGAGCCTCTACTCTC-3’</td>
<td>Annealing: 60°C</td>
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</tr>
<tr>
<td>IL-1β</td>
<td>Sense: 5’-AAGGGAACCAAGGAGCGAC-3’</td>
<td>34 cycles</td>
<td>381</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-GAGATTGAGCTGTCTGCTCA-3’</td>
<td>Annealing: 63°C</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>Sense: 5’-GCTGTCAATGCCTGAAGACC-3’</td>
<td>34 cycles</td>
<td>308</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-GGCTGTGGACAACCAACGCC-3’</td>
<td>Annealing: 63°C</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>Sense: 5’-CCTTGATCCAGGGTGATGAGC-3’</td>
<td>34 cycles</td>
<td>374</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-TTGACCTCAGCGCTGAGTTG-3’</td>
<td>Annealing: 65°C</td>
<td></td>
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<td>MIP-2</td>
<td>Sense: 5’-GCTTGAGAGCCTCTACTCTC-3’</td>
<td>28 cycles</td>
<td>189</td>
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<tr>
<td></td>
<td>Antisense: 5’-TAGTCCCAACTACCTCCCTC-3’</td>
<td>Annealing: 65°C</td>
<td></td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Sense: 5’-ACTGCCCTTGCTGTCTCTCT-3’</td>
<td>34 cycles</td>
<td>261</td>
</tr>
<tr>
<td></td>
<td>Antisense: 5’-AGGCCATTAGTTCCAGTCTA-3’</td>
<td>Annealing: 61°C</td>
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</tr>
</tbody>
</table>
which served as controls, were anesthetized, shaved, and exposed to 24°C water. After sham or burn injury, mice were resuscitated with 1 ml of 0.9% sterile normal saline solution by i.p. injection and were individually housed. L-703606, (cis-2-(diphenylmethyl)-N-[2-(iodophenyl)ethyl]-1-azabicyclo[2.2.2]octan-3-amine oxalate salt) (Sigma-Aldrich), or saline was given i.p. to mice at a dose of 12 mg/kg 1 h before sham or burn injury. L-703606 binds with high affinity to NK1R and has been shown to be a potent antagonist to it (20–22). In preliminary experiments, a dose of 4–12 mg/kg of L-703606 administered 1 h before burn injury was found to significantly inhibit lung neutrophil accumulation and was used for all future experiments in this study (data not shown). One and 8 h after sham or burn injury, animals were sacrificed by an i.p. injection of a lethal dose of pentobarbitone (90 mg/kg). The blood was collected by cardiac puncture and plasma was prepared from anticoagulated blood. Samples of lung and plasma were collected from the animals. Lung samples were homogenized in 1 ml of ice-cold SP assay buffer for 20 s (Bachem/Peninsula Laboratories). The homogenates were centrifuged (13,000 × g, 20 min, 4°C) and the supernatants collected. They were stored at −80°C for subsequent analysis.

Measurement of SP levels

Samples of lung and plasma were collected from the animals. Lung samples were homogenized in 1 ml of ice-cold SP assay buffer for 20 s (Bachem/Peninsula Laboratories). The homogenates were centrifuged (13,000 × g, 20 min, 4°C) and the supernatants collected. They were adsorbed on C18 cartridge columns (Bachem) as described (18, 23). The adsorbed peptide was eluted with 1.5 ml of 75% (v/v) acetonitrile. The samples were freeze-dried and reconstituted in the SP assay buffer (Bachem/Peninsula Laboratories). SP content in the samples was then determined with an ELISA kit (Bachem/Peninsula Laboratories) according to the manufacturer’s instructions and expressed as nanograms per milliliter. Results were then corrected for the DNA content of the tissue samples fluorometrically using Hoechst dye 33258 (24) and were expressed as nanograms per microgram of DNA.

Measurement of myeloperoxidase (MPO) activity

Neutrophil sequestration in lung was quantified by measuring tissue MPO activity. Tissue samples were thawed, homogenized in 20 mM phosphate buffer (pH 7.4), centrifuged (13,000 × g, 10 min, 4°C), and the resulting pellet was resuspended in 50 mM phosphate buffer (pH 6.0) containing 0.5% (w/v) hexadecyltrimethylammonium bromide (Sigma-Aldrich). The suspension was subjected to four cycles of freezing and thawing and was further disrupted by sonication (40 s). The sample was then centrifuged (13,000 × g, 5 min, 4°C) and the supernatant used for the MPO assay. The reaction mixture consisted of the supernatant (50 µl), 1.6 mM tetramethylbenzidine (Sigma-Aldrich), 80 mM sodium phosphate buffer (pH 5.4), and 0.3 mM hydrogen peroxide (reagent volume: 50 µl). This mixture was incubated at 37°C for 110 s, the reaction terminated with 50 µl of 0.18 M H2SO4, and the absorbance measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue sample and results were expressed as fold increase over control (24).

Measurement of pulmonary microvascular permeability

Two hours before sacrifice, each animal received an i.v. bolus injection containing FITC-albumin (5 mg/kg; Sigma-Aldrich) dissolved in normal saline. After mice were euthanized by an i.p. injection of pentobarbitone (90 mg/kg), the blood was collected by cardiac puncture and plasma was separated. The tracheae were exposed and cannulated and the lungs were
washed twice with 1 ml of saline to provide 2 ml of bronchoalveolar lavage fluid. The lavage fluid was combined and FITC fluorescence was measured in the lavage fluid and plasma (excitation 494 nm; emission 520 nm). The ratio of fluorescence emission in lavage fluid to plasma was calculated and used as a measure of pulmonary microvascular permeability (18, 23).

Histopathological examination
A small portion of lung was excised and fixed with 10% neutral buffered formaline (Sigma-Aldrich), dehydrated, and embedded in paraffin for routine histology. Sections of 5-μm thickness were stained with H&E and examined by light microscopy at 200 magnification.

White blood corpuscles-differential count
Blood was collected by cardiac puncture into EDTA-containing tubes at each time point. Samples were analyzed by Cell-Dyn 3700 (Abbott Laboratories) for white blood corpuscles-differential counts.

Cytokines and chemokines analysis
For the measurement of cytokines (IL-1β, IL-6, and TNF-α) and chemokines (MIP-1α and MIP-2) in homogenized lung, ELISA kits from R&D Systems were used according to the manufacturer’s instructions. The lower limits of detection of the levels of IL-1β, IL-6, TNF-α, MIP-1α, and MIP-2 were 15.625, 15.625, 31.25, 3.91, and 15.625 pg/ml, respectively. The ELISA results were reproducible with interassay variability of <9.5% and intraassay variability of <6.5%. Results were then corrected for the DNA content of the tissue samples (24) and were expressed as picograms per microgram of DNA.

Semi-quantitative RT-PCR analysis
Total RNA from lung was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. One microgram of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) at 25°C for 5 min, followed by incubation at 42°C for 30 min, and finally at 85°C for 5 min. The cDNA was then used as a template for PCR amplification by iQ Supermix (Bio-Rad). The primer sequences for detection of PPT-A, NK1R, IL-1β, IL-6, TNF-α, MIP-1α, MIP-2, and 18S, optimal annealing temperature, optimal cycles, and product sizes are shown in Table I. PCR amplification was conducted in MyCycler (Bio-Rad). The reaction mixture was first subjected to 95°C for 3 min, followed by an optimal cycle of amplifications, consisting of 95°C for 30 s, optimal annealing temperature (Table I) for 30 s, and 72°C for 30 s. The optimal annealing temperature (Table I) for 30 s, optimal annealing temperature (Table I) for 30 s, and 72°C for 30 s. Final extension was at 72°C for 10 min. PCR products were analyzed on 1.5% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide.
Statistics

The data were expressed as means ± SEM. The significance of difference among groups was evaluated by ANOVA with a posthoc Tukey’s test for multiple comparisons. A value of \( p < 0.05 \) was regarded as statistically significant.

Results

Burn injury augments significant expression of SP and biological activity of SP-NK1R signaling

The concentration of SP was significantly increased early after burn injury as compared with sham-injured mice at 1 and 8 h in both lung (Fig. 1A, \( p < 0.01 \)) and plasma (Fig. 1B, \( p < 0.01 \)). SP levels were observed to exhibit a trend of higher levels at 8 h compared with 1 h in both lung and plasma. Densitometric analysis of PCR products on agarose gel showed that \( \text{PPT-A} \) mRNA expression, at both 1 and 8 h, correlated well with protein expression levels, with 8 h showing a further rise in levels compared with 1 h (Fig. 2A, \( p < 0.05 \) at 1 h and \( p < 0.01 \) at 8 h). Additionally, pulmonary mRNA levels of \( \text{NK1R} \) expression were concurrently elevated (Fig. 2B, \( p < 0.01 \)) as \( \text{PPT-A} \) mRNA was increased as early as 1 h after burn injury. \( \text{NK1R} \) mRNA levels were markedly increased at 1 h, but decreased to basal levels at 8 h after burn injury.

We also investigated the link between SP and NK1R signaling at both the protein and transcriptional levels after burn injury. Administration of L-703606, a specific NK1R antagonist at 12 mg/kg, significantly suppressed the protein levels of SP in both lung (Fig. 1A, \( p < 0.01 \) at 1 h and \( p < 0.05 \) at 8 h) and plasma (Fig. 1B, \( p < 0.01 \) at 1 and 8 h). Likewise, transcriptional levels of \( \text{PPT-A} \) and \( \text{NK1R} \) gene expression were markedly reduced upon pretreatment with L-703606 (Fig. 2A, \( p < 0.01 \) at 1 h, \( p < 0.05 \) at 8 h, and Fig. 2B, \( p < 0.01 \) at 1 h). A greater attenuation of \( \text{PPT-A} \) and \( \text{NK1R} \) levels occurred at 1 h in both cases, but at 8 h only \( \text{PPT-A} \) expression was suppressed and there was no difference in \( \text{NK1R} \) mRNA expression by 8 h. Taken together, these results indicate that SP biological activity and transcriptional activity after burn injury is NK1R signaling dependent in both lung tissue and plasma.

The enhanced SP-NK1R response correlates with exacerbated lung damage after burn, and disruption of SP-NK1R signaling by L-703606 reverses this effect

The clinical pathology of acute lung injury includes a marked influx of polymorphonuclear leukocytes with increased microvascular permeability and edema (18). Therefore, to determine whether SP-NK1R signaling is critical in mediating burn injury-induced

| FIGURE 5. Effect of L-703606 on cytokine and chemokine mRNA levels in lung early after burn injury. Mice were randomly given L-703606 (12 mg/kg, i.p.) or vehicle (saline) 1 h before burn injury. Sham burn mice served as controls. The levels of TNF-α (A), IL-1β (B), IL-6 (C), MIP-2 (D), and MIP-1α (E) mRNA in lung was determined 1 and 8 h postburn by semiquantitative RT-PCR (determined as ratio of band densities of TNF-α, IL-1β, IL-6, MIP-2, or MIP-1α to 18S) as described in Materials and Methods. Mouse 18S served as a control. Results shown are the mean values ± SEM (\( n = 6 \) mice/group at each time point), using one-way ANOVA and Tukey’s test for multiple comparisons. *, \( p < 0.05 \) vs sham; **, \( p < 0.01 \) vs sham; #, \( p < 0.05 \) vs burn + saline; ##, \( p < 0.01 \) vs burn + saline. |
The pulmonary inflammatory response to injury encompasses the release of large quantities of proinflammatory cytokines, termed a "cytokine storm", which then sets in motion the downstream phenotypic changes in host response after injury (9, 12). Therefore, to relate SP-NKIR signaling in generating a cytokine-mediated lung pathology after burn, we investigated the role of SP-NKIR signaling in affecting these mediators at the protein and transcriptional level.

Table II. Hematologic analysis of whole blood samples from sham- and burn-injured mice

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>White Blood Cells</th>
<th>Neutrophils</th>
<th>Monocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 h postburn</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.27 ± 0.10</td>
<td>0.61 ± 0.03</td>
<td>0.02 ± 0.01</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>Sham</td>
<td>1.33 ± 0.09</td>
<td>0.63 ± 0.04</td>
<td>0.02 ± 0.01</td>
<td>0.68 ± 0.08</td>
</tr>
<tr>
<td>Burn + saline</td>
<td>2.71 ± 0.41bc</td>
<td>1.16 ± 0.10</td>
<td>0.07 ± 0.02</td>
<td>1.68 ± 0.26b</td>
</tr>
<tr>
<td>Burn + L-703606 (12 mg/kg)</td>
<td>1.56 ± 0.12d</td>
<td>0.71 ± 0.05c</td>
<td>0.03 ± 0.01d</td>
<td>0.83 ± 0.09d</td>
</tr>
<tr>
<td>8 h postburn</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>1.29 ± 0.09</td>
<td>0.60 ± 0.04</td>
<td>0.02 ± 0.01</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>Sham</td>
<td>1.35 ± 0.10</td>
<td>0.59 ± 0.04</td>
<td>0.02 ± 0.01</td>
<td>0.62 ± 0.06</td>
</tr>
<tr>
<td>Burn + saline</td>
<td>4.20 ± 0.21b</td>
<td>2.57 ± 0.18b</td>
<td>0.05 ± 0.01b</td>
<td>1.72 ± 0.20b</td>
</tr>
<tr>
<td>Burn + L-703606 (12 mg/kg)</td>
<td>3.22 ± 0.13c</td>
<td>1.69 ± 0.16c</td>
<td>0.03 ± 0.02d</td>
<td>0.59 ± 0.09c</td>
</tr>
</tbody>
</table>

* Mice were randomly given L-703606 (12 mg/kg, i.p.) or vehicle (saline) 1 h before burn. Anticoagulated whole blood at 1 and 8 h postburn was analyzed for percentage neutrophils, monocytes, and lymphocytes as described in Materials and Methods (n = 8–12 mice/group per time point). Data are means ± SEM, compared by one-way ANOVA and Tukey’s test for multiple comparisons.

b p < 0.01, burn + saline vs sham.

p < 0.05, burn + saline vs burn + L-703606 (12 mg/kg).

p < 0.05, burn + saline vs burn + L-703606 (12 mg/kg).

p < 0.05). Therefore, the protective effect of L-703606 is mediated by blockage of SP-induced vascular permeability that may otherwise lead to pulmonary edema and hypoxemia.

Histological examination of lung tissue revealed significantly higher alveolar congestion, a marked increase in perivascular and interstitial inflammatory cellular infiltrates, interstitial edema, and widening of alveolar septa in untreated mice at 1 and 8 h postburn. Pretreatment with L-703606 significantly restored normal lung histology at both 1 and 8 h after burn injury, as observed in normal and sham-injury mice (Fig. 3, C–J).

Burn injury-induced SP-NKIR signaling enhanced gene expression and production of proinflammatory cytokines and chemokines

The pulmonary inflammatory response to injury encompasses the release of large quantities of proinflammatory cytokines, termed a “cytokine storm”, which then sets in motion the downstream phenotypic changes in host response after injury (9, 12). Therefore, to relate SP-NKIR signaling in generating a cytokine-mediated lung pathology after burn, we investigated the role of SP-NKIR signaling in affecting these mediators at the protein and transcriptional level.

Table III. Percentage of leukocyte subsets in circulating blood from sham- and burn-injured mice

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Percentage Leukocyte Subset (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Neutrophils</td>
</tr>
<tr>
<td>1 h postburn</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>23.44 ± 1.05</td>
</tr>
<tr>
<td>Sham</td>
<td>28.51 ± 2.48</td>
</tr>
<tr>
<td>Burn + saline</td>
<td>56.15 ± 1.32b</td>
</tr>
<tr>
<td>Burn + L-703606 (12 mg/kg)</td>
<td>41.15 ± 2.11d</td>
</tr>
<tr>
<td>8 h postburn</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>22.97 ± 0.97</td>
</tr>
<tr>
<td>Sham</td>
<td>26.48 ± 2.02</td>
</tr>
<tr>
<td>Burn + saline</td>
<td>77.56 ± 5.31b</td>
</tr>
<tr>
<td>Burn + L-703606 (12 mg/kg)</td>
<td>61.12 ± 4.27c</td>
</tr>
</tbody>
</table>

* Mice were randomly given L-703606 (12 mg/kg, i.p.) or vehicle (saline) 1 h before burn. Anticoagulated whole blood at 1 and 8 h postburn was analyzed for percentage neutrophils, monocytes, and lymphocytes as described in Materials and Methods (n = 8–12 mice/group per time point). Data are means ± SEM, compared by one-way ANOVA and Tukey’s test for multiple comparisons.

b p < 0.01, burn + saline vs sham.

p < 0.05, burn + saline vs sham.

p < 0.05, burn + saline vs burn + L-703606 (12 mg/kg).

p < 0.05, burn + saline vs burn + L-703606 (12 mg/kg).
We observed markedly increased IL-1β (p < 0.01), TNF-α (p < 0.05), and IL-6 (p < 0.01) protein expression at both 1 and 8 h in lung homogenates, all of which were significantly inhibited (p < 0.01) by pretreatment of L-703606, except for IL-6, which showed a reduction in levels only at 8 h postburn but not at 1 h (Fig. 4, A–C). At the transcriptional level, a similar trend was observed that correlated well with the protein levels showing significant increase in IL-1β (p < 0.05), TNF-α (p < 0.05), and IL-6 (p < 0.01) mRNA, and these were suppressed (p < 0.05) upon L-703606 administration (Fig. 5, A–C). Notably, a marked reduction in IL-6 mRNA levels was found at 1 h, although there was no change at the protein level.

After burn injury, neutrophils and monocytes accumulate in the lungs. To address the role of SP-NK1R signaling on chemotactant production in mediating inflammatory cells, chemokine induction after burn was next investigated. We have assessed pulmonary neutrophil sequestration as an early marker of pulmonary inflammation by determining MPO activity; therefore, it was appropriate to examine the expression of MIP-2, a strong neutrophil chemotactant produced mainly by alveolar macrophages (25).

High levels of MIP-2 after burn injury at 1 and 8 h (Fig. 4D, p < 0.01) correlated well with elevated levels of MPO activity. Likewise, disruption of SP-NK1R signaling by L-703606 suppressed MIP-2 levels (Fig. 4D, p < 0.01 at 1 h and p < 0.05 at 8 h) and, as such, a reduction in MPO activity was observed at both 1 and 8 h as well. MIP-1α is a product of activated monocytes and macrophages and also an important activator of T cells, monocytes, and macrophages (25). Here, L-703606 significantly prevented MIP-1α expression (p < 0.01 at 1 h, p < 0.05 at 8 h) compared with untreated mice (p < 0.05) at both 1 and 8 h (Fig. 4E). Transcriptional levels of MIP-2 and MIP-1α correlated well with the protein levels with (p < 0.05) and without (p < 0.01) L-703606 administration, except that MIP-1α mRNA at 8 h was no longer elevated (Fig. 5, D and E).

Effect of SP-NK1R signaling on immune cells and platelets in circulatory population after burn injury

We have found high levels of SP in plasma after burn and that SP was greatly reduced by L-703606 (Fig. 1B). Therefore, further effort to determine the effect of SP-NK1R signaling on immune cells such as neutrophils, lymphocytes, monocytes, and platelets in circulation would substantiate its role in mediating inflammation after burn.

Absolute numbers of white blood cell, neutrophil, monocyte, and lymphocyte count, including percentage of neutrophil and monocyte subsets, all exhibited a significant increase in the circulation after burn as compared with sham-injured mice (Tables II and III). However, blockage of SP-NK1R signaling by L-703606 treatment significantly reduced all these levels. More interestingly, absolute platelet count was significantly decreased (p < 0.01 at 1 and 8 h) after burn injury, but treatment with L-703606 led to significant improvement (p < 0.01) in blood platelet levels (Fig. 6).

Discussion

There is increasing evidence that neuropeptides and their receptors are important mediators of inflammation via sensory innervation and neurogenic inflammatory events, leading to the development and progression of multiple inflammatory diseases (1, 2). This phenomenon, termed “neurogenic inflammation”, refers to inflammatory responses that result from the release of molecules from primary sensory nerve fibers (26). SP, in particular, has been established to exert a broad range of proinflammatory neural effects in vitro and in vivo, influencing numerous immune and nonimmune effector cell types (27, 28).

In previous studies of neurogenic inflammation after burn injury, the effect of SP was only studied in the local cutaneous wound site or hind paw (29–34). None has addressed the role of neuropeptides like SP in inducing distant organ injury, such as lungs, via neurogenic inflammatory events after burn injury. Mortality resulting from multiple end-organ failure has remained the primary cause of death in critically injured individuals, with the lungs frequently being the first organ to fail (35, 36). Pulmonary complications in burn individuals have been reported even when the lungs have not sustained direct thermal damage (37, 38). Once respiratory failure occurs, proven therapeutic interventions are limited (39). Therefore, intense research on elucidating burn-induced pulmonary pathophysiology and potential therapeutic strategies are of great interest.

Results in the present study reveal for the first time that the enhanced release of SP levels in lung and blood could be a critical factor in leading to the development of systemic inflammation and pathogenesis of local burn injury-induced distant lung organ damage due to overstimulation of C-fibers. Data from a series of experiments indicate that burn injury instigates significant elevation of PPT-A gene levels and SP protein release in lung at 1 and 8 h postburn. Furthermore, both SP and PPT-A mRNA were higher at 8 h than at 1 h in lung, and this elevation correlated with higher lung damage at 8 h compared with 1 h, evidenced by an increase in MPO activity, microvascular permeability, and histological examination revealing higher alveolar congestion, interstitial inflammatory cellular infiltrates, and edema.

SP elicits its proinflammatory neurogenic effects via binding to NK1R. Interestingly, after burn injury, NK1R mRNA peaked at only 1 h but returned to basal levels at 8 h although SP, PPT-A, and lung injury levels were significantly higher at 8 h than at 1 h. This raises the question of whether NK1R on lung effector cells has an effect on modulating SP and lung damage. This was confirmed through the use of a selective NK1R antagonist, L-703606, which significantly suppressed SP at the transcriptional and protein levels in lung 1 and 8 h postburn. Therefore, the data suggest that NK1R regulates SP possibly via a mechanism where only blockade of NK1R down-regulates SP and PPT-A gene levels. Additionally, this regulation of NK1R on SP does not depend on the number of NK1R expressed, because SP production, PPT-A levels, and lung damage continued to rise even when NK1R mRNA expression returned to basal levels at 8 h in burn-injured mice group. Taken

FIGURE 6. Effect of L-703606 on platelet count following burn injury. Mice were randomly given L-703606 (12 mg/kg, i.p.) or vehicle (saline) 1 h before burn injury. Sham burn mice served as controls. Platelet counts were determined at 1 and 8 h as described in Materials and Methods. Results shown are the mean values ± SEM (n = 8–12 mice/group at each time point), using one-way ANOVA and Tukey’s test for multiple comparisons. **, p < 0.01 vs sham; ##, p < 0.01 vs burn + saline.
together, these findings show that neurogenic inflammation after burn occurs via biological activity of SP-NK1R signaling, which continues to increase, regardless of the number of NK1R expressed, so long as the receptor complex is intact, invariably intensifying the severity of pulmonary injury; however, once blockade of the NK1 receptor complex occurs, the signaling is disrupted and SP levels and lung damage are reduced.

A diverse spectrum of SP-induced neuroimmune regulatory effects via signaling pathways involving cytokines (40), chemokines (41), reactive oxygen species (42), and other mediators has been described (1). The generation of these mediators is thought to be through hyperstimulation of immunoinflammatory cells via SP abnormally signaling to the NK1 receptors, which may not be involved in the cellular response to SP under normal physiological conditions (1). Also, the implication of SP in hematopoiesis from in vitro studies further substantiates this reasoning, as SP had been demonstrated to stimulate bone marrow progenitors of both erythroid and myeloid lineages (43). Specifically, the immune cell populations characterized include neutrophils (44), eosinophils (44), lymphocytes (5), monocytes (4), macrophages (4), and mast cells (45), all of which express membrane-bound NK1R. Therefore, upon activation of SP-NK1 complex, numerous immunoinflammatory cells are rapidly triggered to release a cascade of proinflammatory mediators, which progress to cause exacerbated pulmonary damage.

Our results reveal the effect of SP-NK1R signaling on such immunoinflammatory cells in the circulation after burn injury. At 1 and 8 h postburn, neutrophils, monocytes, lymphocytes, and total white blood cell count were significantly elevated, and this effect was abolished upon treatment with L-703606. Furthermore, the trend of SP production in plasma at both time points showed a similar correlation: as SP was increased in blood after burn, there was an increase in neutrophils, monocytes, lymphocytes, and total white blood cell count. Likewise, when NK1R was blocked by L-703606, SP levels in plasma were significantly reduced and expressions of immune cells were also down-regulated in blood.

Interestingly, platelets showed an opposite trend in our findings, where a reduction in circulating platelets after burn injury was found but significantly improved after disruption of SP-NK1R signaling. Platelets have been shown to be a store of SP and to express NK1R (46). Recently, tachykinin stimulation of platelets isolated from healthy mice caused platelet aggregation, while blockade by NK1R antagonist reduced thrombus formation (47). However, platelet numbers were reported in another study to decrease following burn injury (48). In our model of burn injury, we see a similar effect of a significant reduction in circulating platelets. Additionally, we observe for the first time that platelet count improves significantly upon blocking of NK1R by L-703606 1 and 8 h postburn. Published clinical observations document that burn patients who undergo episodes of thrombocytopenia correlate with increased mortality (49, 50). However, the mechanisms responsible for the loss of platelets after severe trauma remain unknown (48). Taken together, the data suggest that SP may have a role in contributing to decrease of platelets and in modulating different cell types following burn injury.

The development of heightened inflammation and lung injury associated with leukocyte infiltration in the lungs was observed along with increased proinflammatory IL-1β, TNF-α, and IL-6 mRNA and protein production 1 and 8 h after burn injury in lung. Chemokines, MIP-2, and MIP-1α were markedly increased, suggesting the active role of SP-induced chemotacticant action in mediating trafficking of inflammatory cells. Likewise, levels of cytokines and chemokines were markedly suppressed upon administration of L-703606. These data suggest that SP plays a crucial role in modulating leukocyte recruitment, as seen in MPO results, through chemokines such as MIP-2 and MIP-1α and that the leukocytes recruited may play an active role in mediating release of proinflammatory cytokines that exacerbate lung inflammation and injury.

In summary, our findings provide for the first time important information on SP neural activity in exacerbating pulmonary inflammatory events and injury via NK1R signaling after burn. Additionally, we show that SP can modulate circulating immune cells and platelet expression in burn injury. Furthermore, the results of this study indicate that the inhibition of tachykinin actions may provide a possible form of therapeutic intervention for the prevention of an acute pulmonary inflammatory cascade following severe injury.

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Disclosures
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References


