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*J Immunol* 2010; 185:6265-6276; Prepublished online 6 October 2010; doi: 10.4049/jimmunol.1001739

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
Substance P Upregulates Cyclooxygenase-2 and Prostaglandin E Metabolite by Activating ERK1/2 and NF-κB in a Mouse Model of Burn-Induced Remote Acute Lung Injury

Selena W. S. Sio,* Seah Fang Ang,† Jia Lu,‡ Shabbir Moolchaha,*† and Madhav Bhatia*‡

Acute lung injury (ALI) is a major cause of mortality in burn patients, even without direct inhalational injury. Identification of early mediators that instigate ALI after burn and of the molecular mechanisms by which they work are of high importance but remain poorly understood. We previously reported that an endogenous neuropeptide, substance P (SP), via binding neurokinin-1 receptor (NK1R), heightens remote ALI early after severe local burn. In this study, we examined the downstream signaling pathway following SP-NK1R coupling that leads to remote ALI after burn. A 30% total body surface area full-thickness burn was induced in male BALB/c wild-type (WT) mice, preprotachykinin-A (PPT-A) gene-deficient mice, which encode for SP, and PPT-A−/− mice challenged with exogenous SP. Local burn injury induced excessive SP-NK1R signaling, which activated ERK1/2 and NF-κB, leading to significant upregulation of cyclooxygenase (COX)-2, PGE metabolite, and remote ALI. Notably, lung COX-2 levels were abrogated in burn-injured WT mice by L703606, PD98059, and Bay 11-7082, which are specific NK1R, MEK-1, and NF-κB antagonists, respectively. Additionally, burn-injured PPT-A−/− mice showed suppressed lung COX-2 levels, whereas PPT-A−/− mice injected with SP showed augmented COX-2 levels postburn, and administration of PD98059 and Bay 11-7082 to burn-injured PPT-A−/− mice injected with SP abolished the COX-2 levels. Furthermore, treatment with parecoxib, a selective COX-2 inhibitor, attenuated proinflammatory cytokines, chemokines, and ALI in burn-injured WT mice and PPT-A−/− mice injected with SP. To our knowledge, we show for the first time that SP-NK1R signaling markedly elevates COX-2 activity via ERK1/2 and NF-κB, leading to remote ALI after burn. The Journal of Immunology, 2010, 185: 6265–6276.

Respiratory failure is a primary cause of mortality in burn patients, and therapeutic interventions against it continue to be a major healthcare challenge (1–4). In severe cases of burn injury, even in the absence of inhalational injury and infection, the ongoing local burn wound inflammation is a sufficient triggering source to induce systemic inflammation and multiple organ failure (2, 5–7). Particularly, the lungs are frequently the first organ to fail after burn due to the early development of acute lung injury (ALI) and the acute respiratory distress syndrome (ARDS) (2–4). The underlying mechanism behind ALI/ARDS is thought to involve the excessive liberation of early inflammatory mediators that instigate and intensify the pulmonary inflammatory cascade, leading to remote ALI/ARDS (7, 8). However, identification of these inflammatory molecules in the lungs after burn as well as the precise signaling mechanisms by which they work are not well understood.

Substance P (SP) is an 11-aa neuropeptide that serves as a major mediator in bidirectional cross-talk between the nervous and immune systems (9). It is encoded by the preprotachykinin-A (PPT-A) gene, is produced by sensory nociceptive neurons and numerous immune cells, and has been shown to play a proinflammatory role in many respiratory diseases (9, 10). Previously, we have reported that SP via binding to the neurokinin-1 receptor (NK1R) contributes significantly to the pathophysiology of remote ALI early after severe local burn (11, 12). However, the downstream signaling pathway following SP-NK1R coupling that leads to remote ALI after burn remains unknown.

PGE2 is a potent lipid mediator produced in nearly every cell type where it is synthesized from arachidonic acid via the actions of cyclooxygenase (COX) enzymes, COX-1 and COX-2 (13). COX-1 is constitutively expressed and is important for normal regulation of vascular activity and gastrointestinal function. COX-2 is an inducible enzyme stimulated by numerous mitogens, cytokines, oxidants, and microbial products. Importantly, COX-2 is primarily responsible for the synthesis of PGE2 (14). COX-2 elevates inflammation by increasing vasodilation, vascular permeability, and edema in numerous respiratory diseases, such as ALI, chronic obstructive pulmonary disease, pulmonary fibrosis, and lung cancer (15–18). Some studies suggest that COX-2 and PGE2 might be associated with SP-related inflammatory responses. For example, in vitro studies have shown SP to incite COX-2 and PGE2 expression via the JAK–STAT pathway in human colonic epithelial cells (19), and via the MAPK pathway in HUV-ECs (20). SP was also shown to induce COX-2 expression and NF-κB activation in human polymorphonuclear leukocytes (21). Additionally, SP stimulated PGE2 via NK1R in isolated rat intrapulmonary bronchi and trachea preparations (22, 23) and via PGE2R subtype 2 in murine tracheal...
preparations (24). However, the interactions between SP, COX-2, and PGE₂ in inducing remote ALI after severe local burn are not known. Therefore, in the present study we investigated whether SP can upregulate COX-2 and work in conjunction with it to instigate remote ALI early after severe local burn, as well as the molecular pathway by which this occurs.

Materials and Methods

Mouse burn injury model

All experiments were approved by the Institutional Animal Care and Use Committee of DSO National Laboratories and conducted in accordance with their established guidelines. Burn injury was performed as previously described (11, 12, 25). Briefly, male BALB/c mice, 6–8 wk old, were anesthetized with ketamine and xylazine (160 and 4 mg/kg, respectively, i.p.) 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 (25). Sham mice, which served as controls, were anesthetized, shaved, and exposed to 24°C room temperature water. After sham or burn injury, mice were resuscitated with 1 ml of 0.9% sterile saline (i.p.) and were individually housed. Two hours after sham or burn injury, animals were sacrificed by a lethal dose of pentobarbitone (90 mg/kg, i.p.). Samples of lung were collected and stored at −80°C for subsequent analysis.

PPT-A+/− mice were a gift from Prof. A. Bashbaum (University of California, San Francisco, CA) and bred as described previously (26). Groups of male PPT-A+/− mice with BALB/c background and their wild-type (WT), PPT-A+/− BALB/c male mice, were randomly selected for sham or burn injury. In a separate group, SP (0.12 µg/kg, i.v.; Bachem/Peninsula Laboratories, San Carlos, CA) dissolved in 0.9% sterile saline was exogenously administered to PPT-A+/− mice (11). Notably, the SP dose of 0.12 µg/kg was established as previously described (11).

In some WT mice, L703606 (12 mg/kg, i.v.; Sigma-Aldrich, St. Louis, MO), a potent antagonist of NK1R (27, 28), or vehicle (0.9% sterile saline) was administered 1 h before sham or burn injury (12). In a separate group, some WT mice were treated with parecoxib (Dyax Inc.; Pizer, New York, NY), a water-soluble, potent, and selective COX-2 inhibitor (29–31). Parecoxib was dissolved in 0.9% sterile saline immediately before use and administered at several concentrations (0, 5, 10, 20, and 30 mg/kg, i.v) 20 min after sham or burn injury. A final dose of 30 mg/kg parecoxib was chosen for all future experiments. In another group, PPT-A+/− mice injected with SP (0.12 µg/kg, i.v.) or vehicle (0.9% sterile saline) immediately after sham or burn injury were subsequently administered parecoxib (30 mg/kg, i.v.) 20 min after burn. Additionally, some mice received PD98059 (10 mg/kg, i.p.; Calbiochem, San Diego, CA), a potent and selective antagonist of MEK-1, which is the upstream kinase of ERK1/2 (32). PD98059 was dissolved in 0.5% DMSO in 0.9% sterile saline and administered 1 h before sham or burn injury in WT or PPT-A+/− mice. Bay 11-7082 (20 mg/kg, i.p.; Calbiochem), a specific inhibitor of NF-κB (39), or vehicle (0.5% DMSO in 0.9% sterile saline) was administered 30 min before sham or burn injury in WT or PPT-A+/− mice. The dose of Bay 11-7082 was chosen based on previous studies (40). The final DMSO concentrations used did not have any effect in our experiments (data not shown) and in other studies (37, 41, 42).

Time course studies in lung tissue after burn injury

In separate groups, some WT mice were sacrificed at 0, 0.5, 1, 2, 4, and 8 h after sham or burn injury. Lung samples were collected and stored at −80°C for subsequent measurements of SP levels, COX-2 expression and activity levels, ERK1/2 activation, and IκBα phosphorylation and degradation expression levels. A final time point of 2 h after induction of sham or burn injury was chosen for all future experiments.

Measurement of SP levels

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. The supernatants were then desorbed on C18 cartridge columns (Bachem/Peninsula Laboratories) as previously described (11). The desorbed 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 determined with an ELISA kit (Bachem/Peninsula Laboratories) according to the manufacturer’s instructions. Results were then corrected for the DNA content of the tissue samples fluorometrically using Hoechst dye 33258 (43) and were expressed as nanograms per microgram of DNA.

Measurement of myeloperoxidase activity

Lung neutrophil sequestration was quantified by measuring myeloperoxidase (MPO) activity (11). Lung 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% 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 was terminated by the addition of 50 µl of 2.5 M H₂SO₄. The absorbance at 450 nm was recorded.

FIGURE 1. Time course study of lung SP and COX-2 levels in WT mice after burn. A, SP levels measured by ELISA; B, COX-2 expression levels determined by Western blot, and C, COX-2 activity levels in lung of WT mice at 0, 0.5, 1, 2, 4, and 8 h postburn. At 0 h, sham-injured mice were used as controls. For COX-2 expression evaluation, a representative Western blot image is shown, with densitometry data expressed as average ratios of COX-2 to HPRT levels (plotted as fold increase over control at 0 h). Results shown are the mean values ± SEM (n = 4–6 mice/group for SP levels, 4 mice/group for COX-2 expression levels, and 5–7 mice/group for COX-2 activity levels). *p < 0.05; **p < 0.01 when compared with control group at 0 h.
with 50 μl of 0.18 M H₂SO₄, and the absorbance was measured at 450 nm. This absorbance was then corrected for the DNA content of the tissue samples and results were expressed as fold increase over control (43).

**Histopathological examination**

A small portion of lung was excised and fixed with 10% neutral buffered formalin (Sigma-Aldrich) and then subsequently dehydrated through a graded ethanol series, embedded in paraffin wax, and sectioned as previously described (11). Sections of 5-μm thickness were stained with H&E and examined by light microscopy using a Carl Zeiss light microscope (Thornwood, New York, NY) (×200 magnification).

**Cytokine and chemokine analysis**

Single-analyte ELISA assays were performed for the measurement of cytokines (IL-1β, IL-6, and TNF-α) and chemokines (MIP-1α and MIP-2) in homogenized lung tissue, according to the manufacturer’s instructions (all from R&D Systems, Minneapolis, MN). The lower limits of detection for 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 <0.5% and intra-assay variability of <6.5%. Results were then corrected for the DNA content of the tissue samples (43) and were expressed as picograms per microgram of DNA.

**Measurement of COX-2 activity**

Lung COX-2 activity was measured according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI). Briefly, lung samples were homogenized in ice-cold lysis buffer (0.1 M Tris-HCl [pH 7.5], supplemented with protease inhibitor mixture; Sigma-Aldrich) for 20 s. The homogenates were centrifuged (10,000 × g, 15 min, 4°C) and the supernatants collected for analysis. COX-2 activity was expressed as a percentage of total COX activity.

**Measurement of PGE₂ metabolite levels**

Lung PGE₂ metabolite (PGEM) levels were determined by ELISA kits (Cayman Chemical) according to the manufacturer’s instructions. The results were corrected for the DNA content of the tissue sample and expressed as fold increase over control (43).

**Nuclear extraction and measurement of NF-κB activation**

Nuclear extracts from lung were prepared by using a nuclear extraction kit as described by the manufacturer (Active Motif, Carlsbad, CA). Protein concentrations in nuclear extracts were determined using a Bradford assay (Bio-Rad, Hercules, CA). To monitor NF-κB activation in lung tissues, we used a TransAM NF-κB p65 transcription factor assay kit (Active Motif). The kit consists of a 96-well plate, into which oligonucleotide containing the NF-κB consensus site (5′-GGGACTTTCC-3′) is bound. The active form of NF-κB in the nuclear extract specifically binds to this consensus site and is recognized by a primary Ab specific for the activated form of p65 of NF-κB. An HRP-conjugated secondary Ab provides the basis for the colorimetric quantification. The absorbance of the resulting solution was measured 2 min later (450 nm with a reference wavelength of 655 nm), using a 96-well microplate reader (Tecan Systems, Durham, NC). The wild-type consensus oligonucleotide was provided as a competitor for NF-κB binding to monitor the specificity of the assay. Results were expressed as fold increase over the control group.

**Western immunoblot**

Lungs were homogenized at 4°C in radioimmunoprecipitation assay lysis buffer supplemented with protease and phosphatase inhibitor mixture (Sigma-Aldrich). The tissue homogenates were centrifuged at 14,000 rpm, 10 min at 4°C and the supernatants collected for analysis. COX-2 activity was expressed as a percentage of total COX activity.

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**Western immunoblot**

Lungs were homogenized at 4°C in radioimmunoprecipitation assay lysis buffer supplemented with protease and phosphatase inhibitor mixture (Sigma-Aldrich). The tissue homogenates were centrifuged at 14,000 × g for 10 min at 4°C. Protein concentration in the soluble fraction was determined by the Bradford method. Protein samples (80 μg) were separated...
by SDS-PAGE on Novex 10% Tris-glycine polyacrylamide gels (Invitrogen, Carlsbad, CA) and transferred onto polyvinylidene difluoride membranes (Invitrogen) by electroblotting in Novex transfer buffer (Invitrogen) containing 20% (v/v) methanol. Membranes were then washed, blocked, and probed overnight at 4°C with rabbit anti-COX-2 (Cayman Chemical; 1/1000 dilution), rabbit anti-IκBα, phospho-IκBα, ERK1/2, and phospho-ERK1/2 (Cell Signaling Technology, Beverly, MA; 1/1000 dilution for all), followed by secondary detection for 2 h with an HRP-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA; 1/1000 dilution). Membranes were then washed and then incubated in SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL) before exposure to x-ray films (CL-XPosure; Pierce). Gels were calibrated by protein kaleidoscope standards (Bio-Rad). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Santa Cruz Biotechnology; 1/1000 dilution) was applied as an internal control to normalize protein loading. The intensity of bands was quantified using LabWorks image analysis software (UV Products, San Gabriel, CA).

**Statistics**

The data were expressed as mean ± SEM. The significance of difference among groups was evaluated by ANOVA with a posthoc Tukey’s test for multiple comparisons when comparing three or more groups. A value of $p < 0.05$ was regarded as statistically significant.

**Results**

**Concurrent elevations in lung SP and COX-2 levels after burn in WT mice**

Lung SP levels were increased significantly as early as 0.5 h after burn compared with 0 h postburn, which represents mice without burn injury (Fig. 1A; $p < 0.01$). Moreover, the SP levels continued to remain significantly elevated up to 8 h postburn. In an attempt to determine whether COX-2 levels were simultaneously changed...
after burn, we performed Western blot analysis for the same time points. Our results revealed a parallel increase in COX-2 expression levels, which peaked at 1–2 h postburn (Fig. 1B; p < 0.05). A similar profile was observed in lung COX-2 activity levels, with the highest level having been reached at 2 h after burn (Fig. 1C; p < 0.01). Therefore, burn injury induces a concomitant increase in lung SP and COX-2 levels, which are both prominently expressed at 2 h postburn, and thus this time point was chosen for all future experiments.

Dose-dependent effect of parecoxib, a selective COX-2 inhibitor, on lung neutrophil infiltration in WT mice following burn injury

The clinical pathology of ALI is characterized by the invasion of neutrophils into the lungs (8). Neutrophils predominate in the lungs of patients with ALI (44) and in many animal models of ALI (45, 46). A major constituent of neutrophils is the enzyme MPO (47), which is commonly used to quantify neutrophil accumulation. Therefore, to assess the severity of COX-2–mediated ALI after burn, we tested the effect of a potent and selective COX-2 inhibitor, parecoxib, on lung MPO activity levels. Parecoxib is a water-soluble prodrug of valdecoxib and is the first COX-2–specific inhibitor used for parenteral administration (30, 31). It does not affect COX-1 at therapeutic doses, which is crucial for maintaining normal function in the stomach and blood (29). Parecoxib was administered therapeutically to WT mice at 20 min postburn in a dose-dependent manner of 0, 5, 10, 20, and 30 mg/kg, i.v., followed by measurement of MPO activity at 2 h postburn. Our results revealed a significant reduction in lung MPO activity with 30 mg/kg parecoxib after burn, and therefore this dose was used for all future experiments (Fig. 2; p < 0.01).

Blockade of SP-NK1R signaling and COX-2 significantly protects against burn-induced ALI

Lung MPO activity levels were heightened in WT mice at 2 h postburn (Fig. 3A), which correlated with augmented SP and COX-2 expression levels (Fig. 1). Notably, the MPO activity levels were significantly attenuated in burn-injured WT mice treated with L703606, a specific NK1R antagonist (27, 28), at 12 mg/kg, i.p., given 1 h before burn injury, and parecoxib, administered at 30 mg/kg, i.v., 20 min postburn, suggesting that SP-NK1R signaling and COX-2 play a pivotal role in modulating ALI after burn (Fig. 3A; p < 0.05 for both antagonists). To further confirm

FIGURE 5. SP-NK1R signaling and COX-2 expression leads to increased lung PGEM levels after burn. PGEM levels were measured by ELISA at 2 h in lung of WT, PPT-A−/−, and PPT-A+/− mice injected with SP (0.12 μg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT mice, L703606 (12 mg/kg, i.p.) was administered 1 h before burn injury, or parecoxib (30 mg/kg, i.v.) was administered 20 min postburn. PPT-A−/− mice injected with SP were also subsequently administered parecoxib (30 mg/kg, i.v.) at 20 min postburn. Sham plus saline (WT) mice were used as controls (plotted as fold increase over control). Results shown are the mean values ± SEM (n = 5–8 mice/group). *p < 0.05; **p < 0.01.

FIGURE 6. Time course study of ERK1/2 activation and IkBα phosphorylation and degradation in lungs of WT mice after burn. A, Phospho-ERK, (B) phospho-IkBα, and (C) IkBα expression levels, measured by Western blot, in lung of WT mice at 0, 0.5, 1, 2, 4, and 8 h postburn. A representative Western blot image is shown for each protein, with densitometry data expressed as average ratios of phospho-ERK to total ERK, phospho-IkBα to HPRT, and IkBα to HPRT levels. At 0 h, sham-injured mice were used as controls (plotted as fold increase over control at 0 h). Results shown are the mean values ± SEM (n = 4 mice/group). *p < 0.05; **p < 0.01 when compared with control group at 0 h.
this, we used PPT-A^{−/−} mice. Our results showed that MPO activity levels were significantly decreased in burn-injured PPT-A^{−/−} mice compared with burn-injured WT mice, indicating that when SP levels are augmented, this leads to a significant increase in MPO activity levels after burn (Fig. 3A). Additionally, when SP was exogenously administered to burn-injured PPT-A^{−/−} mice, the MPO activity levels were elevated, whereas alleviated levels of MPO activity were restored upon treatment with parecoxib in burn-injured PPT-A^{−/−} mice injected with exogenous SP (p < 0.05). These results confirm the important role of SP and COX-2...
in instigating lung neutrophil infiltration after burn. Subsequently, we performed histopathological examination at 2 h postburn. Consistently, alleviated alveolar congestion, lowered leukocyte infiltration, reduced alveolar septal wall thickness, and interstitial edema were observed in burn-injured WT mice treated with L703606 and parecoxib and in burn-injured PPT-A−/− mice, compared with burn-injured WT mice (Fig. 3B). Burn-injured PPT-A−/− mice injected with SP demonstrated severe ALI. In contrast, treatment with parecoxib in burn-injured PPT-A−/− mice injected with SP showed significant protection from ALI and restoration of normal lung histopathology (Fig. 3B). Importantly, levels of these cytokines and chemokines were amplified in burn-injured PPT-A−/− mice compared with burn-injured WT mice (Fig. 3B). A representative Western blot image is shown for each protein, with densitometry data expressed as average ratios of phospho-IκBα or IκBα to HPRT levels. Sham plus saline (WT) mice were used as controls (plotted as fold increase over control). Results shown are the mean values ± SEM (n = 4 mice/group). **p < 0.01; ***p < 0.001.

Inhibition of SP-NK1R signaling and COX-2 impaired proinflammatory cytokines and chemokines production after burn

Proinflammatory cytokines IL-1β, IL-6, and TNF-α and chemokines MIP-2 and MIP-1α showed a marked rise in their levels in WT mice at 2 h after burn (Fig. 4). Consistent with MPO and histology results (Fig. 3), burn-injured WT mice treated with L703606 and parecoxib showed basal levels of MPO activity and normal histopathology (Fig. 3), thereby confirming that the doses used had no adverse effect in the lungs, and thus these sham mice administered with L703606 or parecoxib alone were not included in future experiments. Taken together, our findings show that the inhibition of SP-NK1R–mediated expression of COX-2 levels is critical in resolving ALI after burn.

Results show decreased expression of proinflammatory cytokines and chemokines after burn injury when treated with parecoxib. For example, IL-1β, IL-6, and TNF-α levels were significantly reduced in burn-injured PPT-A−/− mice treated with SP compared with burn-injured WT mice (Fig. 3B). Similarly, levels of MIP-2 and MIP-1α were also reduced in burn-injured PPT-A−/− mice treated with SP compared with burn-injured WT mice (Fig. 3B). These findings suggest that SP-NK1R signaling and COX-2 activity occur upstream of PGEM production and can upregulate PGEM levels after burn.

Time course study of lung ERK1/2 activation and IκBα phosphorylation and degradation after burn in WT mice

To investigate the signaling mechanisms by which SP upregulates COX-2 in the lungs, we evaluated ERK1/2, an important component of the MAPK pathway involved in inflammation (54), and IκBα. In agreement with changes over time of increased lung SP and COX-2 expression levels (Fig. 1), ERK1/2 showed augmented phosphorylation at 2 h postburn compared with 0 h (p < 0.01) and remained elevated up to 8 h postburn (Fig. 6A). Furthermore, IκBα phosphorylation rose significantly (Fig. 6B), whereas the content of IκBα was reduced with significant degradation occurring at 2 h postburn (Fig. 6C; p < 0.01). Therefore, local burn injury leads to increased SP-NK1R signaling and COX-2 upregulation.

Increased SP-NK1R signaling and COX-2 levels augmented PGEM production following burn injury

The observed increases in lung COX-2 levels led us to examine lung PGE2 levels after burn. Importantly, PGE2 is rapidly metabolized in vivo (48–50), and therefore measurement of its metabolite, PGEM, is commonly used to provide a reliable estimate of actual PGE2 production (51–53). As expected, lung PGEM levels were upregulated 2 h after burn compared with sham-injured WT mice (Fig. 5; p < 0.01), which correlated with augmented COX-2, SP, and ALI levels in WT mice as observed earlier (Figs. 1, 3). Notably, treatment with L703606 and parecoxib abrogated PGEM levels in WT mice at 2 h postburn (Fig. 5). Furthermore, an obvious reduction in PGEM levels was observed in burn-injured PPT-A−/− mice compared with burn-injured WT mice (p < 0.01). However, upon administration of exogenous SP to burn-injured PPT-A−/− mice, PGEM levels rose again (p < 0.01). More importantly, treatment with parecoxib reduced the PGEM levels in burn-injured PPT-A−/− mice injected with SP (p < 0.01). Taken together, our results show compelling evidence that SP-NK1R signaling and COX-2 activity occur upstream of PGEM production and can upregulate PGEM levels after burn.
injury induces activation of ERK1/2 and IkBa in lung, which were further investigated for the rest of this study.

**Activation of SP-NK1R signaling and ERK1/2 markedly upregulated COX-2 expression levels after burn injury**

To examine the critical link between SP-NK1R signaling and ERK1/2 activation leading to changes in COX-2 expression levels, we performed Western blot analysis for COX-2 expression using L703606 and PD98059. PD98059 is a potent and selective antagonist of MEK-1 that is the upstream kinase of ERK1/2 (32), administered at 10 mg/kg, i.p., 1 h before burn injury. A significant increment in COX-2 expression levels was observed in WT mice at 2 h postburn ($p < 0.05$). However, the COX-2 levels were abolished in burn-injured WT mice treated with both L703606 and PD98059 ($p < 0.05$ for both antagonists). Additionally, usage of burn-injured PPT-A<sup>−/−</sup> mice further substantiated the role of SP in modulating COX-2 expression, whereby burn-injured PPT-A<sup>−/−</sup> mice revealed significantly reduced lung COX-2 levels ($p < 0.05$); however, upon administration of exogenous SP, the COX-2 levels were elevated as anticipated. More significantly, treatment with PD98059 in burn-injured PPT-A<sup>−/−</sup> mice injected with SP markedly suppressed COX-2 expression levels ($p < 0.05$). Therefore, SP-NK1R and ERK1/2 signaling significantly upregulate COX-2 expression levels in the lungs after burn and occur upstream of it.

**Increased SP-NK1R signaling enhanced ERK1/2 activation after burn**

Next, we verified the effect of SP-NK1R signaling on ERK1/2 activity. Our results showed that ERK1/2 had undergone significant phosphorylation at 2 h after burn compared with sham-injured WT mice (Fig. 8; $p < 0.01$). This was decreased in burn-injured WT mice administered with L703606 and PD98059 and in burn-injured PPT-A<sup>−/−</sup> mice. The phosphorylation levels of ERK1/2 were restored in burn-injured PPT-A<sup>−/−</sup> mice injected with SP and again reduced when burn-injured PPT-A<sup>−/−</sup> mice injected with SP were treated with PD98059 (Fig. 8). Therefore, SP-NK1R signaling occurs upstream of ERK1/2, and its inhibition significantly suppresses ERK1/2 activation.

**Effect of SP-NK1R signaling and ERK1/2 activation on IkBa phosphorylation and degradation levels and NF-kB activity after burn injury**

Subsequently, we analyzed whether SP-NK1R and ERK1/2 signaling leads to activation of IkBa and NF-kB after burn injury. Pretreatment with L703606 in burn-injured WT mice drastically reduced the pulmonary levels of phospho-IkBa compared with the heightened levels detected in untreated burn-injured WT mice at 2 h (Fig. 9A; $p < 0.01$). Furthermore, this reduction was consistent in burn-injured WT mice administered with PD98059 and in burn-

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**FIGURE 10.** SP-NK1R signaling and ERK1/2 increases NF-kB activation, which leads to elevated lung COX-2 activity following burn injury. A, The DNA-binding activity of NF-kB in nuclear extracts was measured by ELISA at 2 h in lung of WT, PPT-A<sup>−/−</sup>, and PPT-A<sup>−/−</sup> mice injected with SP (0.12 μg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT mice, L703606 (12 mg/kg, i.p.) or PD98059 (10 mg/kg, i.p.) was administered 1 h before burn injury. PPT-A<sup>−/−</sup> mice injected with SP were also administered PD98059 (10 mg/kg, i.p.) 1 h before burn injury. Sham plus saline (WT) mice were used as controls (plotted as fold increase over control). B, COX-2 activity levels at 2 h in lung of WT, PPT-A<sup>−/−</sup>, and PPT-A<sup>−/−</sup> mice injected with SP (0.12 μg/kg, i.v.) or vehicle (saline) immediately after sham or burn injury. In some WT mice and PPT-A<sup>−/−</sup> mice injected with SP, Bay 11-77082 (20 mg/kg, i.p.) or vehicle was administered 30 min before burn injury. Results shown are the mean values ± SEM ($n = 6–9$ mice/group for NF-kB activity and 3–5 mice/group for COX-2 activity). *$p < 0.05$; **$p < 0.01$.

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injured PPT-A−/− mice. Additionally, the phospho-IκBα levels in burn-injured PPT-A−/− mice injected with SP were again amplified, whereas these levels were suppressed upon treatment with PD98059 (Fig. 9A). Assessment of IκBα degradation displayed a significant reduction in IκBα levels at 2 h after burn in WT mice and in burn-injured PPT-A−/− mice injected with SP, whereas the content of IκBα levels observed in the other groups were significantly higher (Fig. 9B).

The DNA-binding activity of nuclear NF-κB in the lungs of burn-injured WT mice was markedly enhanced compared with sham-injured WT mice, burn-injured WT mice treated with L703606 and PD98059, and burn-injured PPT-A−/− mice (Fig. 10A). Exogenous administration of SP to burn-injured PPT-A−/− mice amplified the activation of NF-κB, whereas pretreatment with PD98059 significantly disrupted the activity of NF-κB.

The increased production of SP after burn leads to NF-κB activation and subsequent elevation in lung COX-2 activity levels

The inducible expression of COX-2 is regulated by various transcription factors (55, 56). Hence, to ascertain if COX-2 is up-regulated by NF-κB after burn, we administered Bay 11-7082 to mice, a specific inhibitor of NF-κB (39), at 20 mg/kg, i.p., 30 min before burn injury, followed by measurement of COX-2 activity levels at 2 h postburn. Both burn-injured WT mice and burn-injured PPT-A−/− mice injected with SP showed increased levels of lung COX-2 activity at 2 h postburn (Fig. 10B). However, pretreatment with Bay 11-7082 showed significantly reduced lung COX-2 activity levels in burn-injured WT mice and in burn-injured PPT-A−/− mice injected with SP (Fig. 10B; p < 0.05). Therefore, our findings collectively show that SP-NK1R signaling activates ERK1/2 and NF-κB, leading to increased COX-2 and remote ALI after severe local burn, while PEGM levels were also concomitantly elevated (Fig. 11).

Discussion

Burn injury is an important global healthcare problem, with respiratory failure commonly listed as one of the major contributors to mortality (1–4). Thus, identifying early endogenous inflammatory molecules and the molecular mechanisms by which they initiate signal transduction pathways that lead to ALI/ARDS after severe burn are of critical importance. Previously, we reported that an endogenous neuropeptide, SP, activates NK1R to instigate remote ALI by increasing lung neutrophil infiltration and lung microvascular permeability, along with disruption of lung function early after severe local burn (11, 12). To our knowledge, in this study we report for the first time that the signaling events following SP-NK1R coupling, which leads to activation of ERK1/2 and NF-κB, followed by COX-2 and PEGM upregulation, significantly elevated proinflammatory cytokines, chemokines, and lung neutrophil invasion and exacerbated remote ALI early after severe local burn in mice.

Results from time course studies showed that the expression levels of lung SP and COX-2 were concomitantly increased after burn. As early as 1 h postburn, SP and COX-2 levels were more than 3-fold higher in WT mice than at 0 h postburn. Similarly, phosphorylation of ERK1/2 and IκBα was increased, along with concurrent IκBα degradation. Taken together, the overproduction of SP and COX-2 along with activation of ERK1/2 and IκBα correlated well and remained elevated in lungs for up to 8 h postburn, thereby suggesting that these cellular changes are not an early temporal rise but are constantly maintained in lung cells for at least up to 8 h postburn.

![FIGURE 11. Schematic summary of signaling events in SP induced remote ALI following severe local burn. The local burn injury induces SP-NK1R signaling, which activates ERK1/2 and NF-κB, leading to upregulation of COX-2 and increased remote ALI. Additionally, lung PEGM levels were concomitantly elevated. Usage of L703606, PD98059, Bay 11-7082, and parecoxib, which are specific NK1R, MEK-1, NF-κB, and COX-2 antagonists, respectively, confirm these signaling events.](https://www.jimmunol.org/content/6273/1/6280)
ERK1/2 and NF-κB, leading to upregulation of COX-2, which instigates remote ALI after local burn. Additionally, results of proinflammatory cytokines and chemokines from burn-injured PPT-A−/− mice injected with exogenous SP and subsequently treated with or without parecoxib revealed that SP can upregulate IL-1β, IL-6, TNF-α, MIP-2, and MIP-1α levels via COX-2. PGE2 is synthesized from arachidonic acid primarily via the actions of COX-2. It is known that >90% of circulating PGE2 is rapidly catabolized to inactive forms on the first pass in the lungs (49, 50, 57). Therefore, studies have used the measurement of PGEM as a common alternative to indicate the levels of PGE2 (51–53, 58, 59). Significantly high levels of PGEM in the lungs were detected in burn-injured WT mice and in burn-injured PPT-A−/− mice injected with SP, which corresponded with concurrent increases in SP-NK1R signaling, COX-2 levels, and ALI. Treatment with L703606 and parecoxib showed significantly reduced levels of PGEM in burn-injured WT mice, while PGEM levels were also lowered in burn-injured PPT-A−/− mice injected with SP and subsequently treated with parecoxib. Furthermore, simultaneous decreases of COX-2 and ALI were also detected in WT mice administered L703606 and parecoxib after burn and in burn-injured PPT-A−/− mice injected with SP and later administered parecoxib. Therefore, SP-NK1R signaling induces PGEM accumulation after burn, which occurred concurrently with increased lung COX-2 expression and exacerbation of ALI. It has been reported in earlier studies that COX-2 levels increased concomitantly with the severity of ALI, whereas inhibition of COX-2 attenuated ALI in an acid model of ALI and carrageenan-induced pleurisy model (60, 61). Additionally, PGE2 has been shown to exert significant immune system dysfunction after burn injury (62, 63). Significant levels of systemic PG were found in the plasma of severely burn-injured patients (64), whereas macrophages isolated from burn-injured experimental animals produced increased COX-2 activity and PGE2 levels (65). Furthermore, it has been reported that inhibition of COX can improve various mononuclear cell-mediated immune responses and survival after burn injury (62, 66). However, the early upstream endogenous molecules capable of instigating the upregulation of COX-2 and PGE2 in the lungs after burn injury are not well understood. Moreover, little information is available on the association of lung MAPK and lung COX-2 expression after burn. Studies have shown that p38 (67–69) and JNK (70, 71) contribute to ALI after burn; furthermore, it has been reported that activation of p38 upregulates COX-2 levels in the gastrointestinal tract after burn (72), but not in the lungs. ERK1/2 has been implicated in many trauma or stress-related injury models, such as ischemic injury (73, 74), hemorrhage (75), sepsis (34), and acute pancreatitis (76). However, none has investigated ERK1/2 and its association with lung COX-2 expression in burn-induced ALI. It has been shown that in vitro stimulation of NK1R by SP in lung epithelial cells results in activation of a Gαq-dependent pathway involving phospholipase C, calcium, protein kinase C, Ras/Raf/ERK1/2, proteasomal degradation of iκB, and NF-κB (77). Additionally, ERK1/2 mediates superoxide production in neutrophils isolated from rats after burn, which are the major effector cells that contribute to ALI (78). However, the detailed molecular mechanisms of burn-induced ALI are not studied comprehensively in vivo. To our knowledge, in our study we show for the first time that SP-NK1R coupling occurs early upstream after burn, which activates ERK1/2 and NF-κB, leading to elevated lung COX-2 expression and remote ALI. Furthermore, lung PGEM levels were concomitantly increased after burn. Collectively, this study may contribute to a better understanding of the precise mechanisms initiated by SP in the pathophysiology of remote ALI early after severe local burn. Additionally, inhibition of SP-NK1R signaling and COX-2 may provide a possible therapeutic approach for the prevention of ALI in critically burn-injured patients.

Acknowledgments

We thank Mei Leng Shoon (Department of Pharmacology, National University of Singapore), Mui Hong Tan, Julie Yeo, David Poon, Cecilia Lim, and Li Li Tan (DSO Laboratories, Singapore) for excellent technical assistance, and Parvathi Rajagopalan (DSO Laboratories, Singapore) for animal care and management. We greatly appreciate Prof. A. Basbaum (University of California, San Francisco) for the gift of PPT-A−/− mice.

Disclosures

The authors have no financial conflicts of interest.

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