Pulmonary changes in a mouse model of combined burn and smoke inhalation-induced injury

Akio Mizutani,1,4 Perenlei Enkhbaatar,1 Aimalohi Esechie,2 Lillian D. Traber,1 Robert A. Cox,3 Hal K. Hawkins,3 Donald J. Deyo,1 Kazunori Murakami,1 Takayuki Noguchi,4 and Daniel L. Traber1,2

Departments of 1Anesthesiology, 2Neuroscience and Cell Biology, and 3Pathology, University of Texas Medical Branch and Shriners Hospital for Children, Galveston, Texas; and 4Department of Anesthesiology and Intensive Care Unit, Oita University Faculty of Medicine, Oita, Japan

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The morbidity and mortality of burn victims increase when burn injury is combined with smoke inhalation. The goal of the present study was to develop a murine model of burn and smoke inhalation injury to more precisely reveal the mechanistic aspects of these pathological changes. The burn injury mouse group received a 40% total body surface area third-degree burn alone, the smoke inhalation injury mouse group received two 30-s exposures of cotton smoke alone, and the combined burn and smoke inhalation injury mouse group received both the burn and the smoke inhalation injury. Animal survival was monitored for 120 h. Survival rates in the burn injury group, the smoke inhalation injury group, and the combined injury group were 70%, 60%, and 30%, respectively. Mice that received combined burn and smoke injury developed greater lung damage as evidenced by histological changes (septal thickening and interstitial edema) and higher lung water content. These mice also displayed more severely impaired pulmonary gas exchange [arterial Po2 (PaO2)/inspired O2 fraction (FiO2)] < 200]. Lung myeloperoxidase activity was significantly higher in burn and smoke-injured animals compared with the other three experimental groups. Plasma NO2/NO3, lung inducible nitric oxide synthase (iNOS) activity, and iNOS mRNA increased with injury; however, the burn and smoke injury group exhibited a higher response. Severity of burn and smoke inhalation injury was associated with more pronounced production of nitric oxide and accumulation of activated leukocytes in lung tissue. The murine model of burn and smoke inhalation injury allows us to better understand pathophysiologic mechanisms underlying cardiopulmonary morbidity secondary to burn and smoke inhalation injury.

Acute lung injury; inducible nitric oxide synthase; nitric oxide

There are over one million cases of burn injury annually in the United States. Although the insult of burn injury alone ranges in severity depending on the temperature and duration of exposure, it is infrequently fatal (22). Acute lung injury [ALI, arterial Po2 (PaO2)/inspired O2 fraction (FiO2) < 300 Torr] or its terminal phase, acute respiratory distress syndrome (ARDS, PaO2/FiO2 < 200 Torr), due to smoke inhalation injury is a serious complication seen in burn patients whose burned area exceeds 30% (22). Thermal injury combined with inhalation injury compromises pulmonary function, producing microvascular hyperpermeability that leads to a significant increase in lung lymph flow and pulmonary edema (9, 30). A well-studied molecule associated with this mechanism is nitric oxide (NO), which has been shown to increase significantly in correlation with increased expression of inducible nitric oxide synthase (iNOS) mRNA in animal studies of ARDS (5). iNOS transcription and activity is induced by cytokines, endotoxins, and other materials (32, 16). Additionally, proinflammatory cytokines such as interleukin (IL)-1α increase in concentration in lung tissue and are known to activate the transcription factor nuclear factor-κB (NF-κB) (12), one of the key transcriptional regulators of iNOS mRNA expression (24), in response to an inflammatory stimulus.

We previously established an ovine model of burn and smoke inhalation to describe the pathophysiology of ALI. We reported (23) that combined burn and smoke inhalation injury produces an increase in plasma NO levels. We also reported that impaired pulmonary function (e.g., poor gas exchange, increased pulmonary transvascular fluid flux, lung water content, histological changes) was associated with increased levels of iNOS mRNA and 3-nitrotyrosine, a stable metabolite of peroxynitrite, in lung tissue taken from sheep after burn injury and smoke insufflation, suggesting an important role of iNOS-derived NO in the pathological process. The ovine model of ALI allows us to mimic a clinical situation through the continuous monitoring of cardiopulmonary variables, long-term study (up to 96 h), acceptance of mechanical ventilation through a tracheostomy without requiring sedation, and an animal with an airway structure that is similar to that of humans, i.e., the presence of bronchial glands. Despite these advantages, it is still not possible to genetically manipulate this model and observe variations in inflammatory response. Therefore, the purpose of the present study was to develop a murine model of burn and smoke inhalation injury to further understand pathophysiologic aspects underlying this serious menace.

MATERIALS AND METHODS

Animal model. This study was approved by the Animal Care and Use Committee of the University of Texas Medical Branch. All animals were handled according to the guidelines established by the American Physiological Society and the National Institutes of Health (NIH).

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Address for reprint requests and other correspondence: D. L. Traber, Investigative Intensive Care Unit, Dept. of Anesthesiology, Univ. of Texas Medical Branch, 610 Texas Ave., Galveston, TX 77555-0833 (e-mail: dltraber@utmb.edu).
Female C57BL/6 mice weighing 20–26 g (4–6 wk of age) were purchased from Jackson Laboratory (Bar Harbor, ME). The animals were anesthetized with 4% vaporized isoflurane in air in a chamber, intubated with a custom-made endotracheal tube (modified from a 20-gauge angiocatheter), and allowed to breathe spontaneously under 2% isoflurane. The backs and flanks of the animals were shaved, and 1.0 ml of 0.9% saline was injected subcutaneously to prevent deep tissue and spinal cord injury by flame burn under anesthesia. The mice were randomized to four groups: 1) a sham injury group that were intubated with no injury, 2) a burn injury group that received a 40% total body surface area (TBSA) third-degree burn, 3) a smoke inhalation injury group that received two 30-s exposures of cool cotton smoke, and 4) a combined burn and smoke inhalation injury group that received both the burn and the smoke inhalation injury.

**Burn and smoke inhalation injury.** Mice were subjected to a 40% TBSA third-degree burn as described previously, with some modification (28). Briefly, the anesthetized mice were covered with a Zetex cloth containing a rectangular opening corresponding to 40% of the mouse TBSA, determined as previously described (29). A full-thickness flame burn was achieved by a Bunsen burner applied to exposed skin for ~10 s. Full-thickness injury was confirmed by loss of coloration and lack of bleeding on incision. Smoke inhalation was induced with a smoker device designed and constructed in our laboratory. The smoker, connected with tubing that provided a constant flow of air, was filled with 20 g of burning cotton toweling and then attached to the custom-made endotracheal tube via a T connection. A copper condenser coil was placed between the smoker and the endotracheal tube to cool down the hot smoke flow. Two sets of 30-s exposure to the cool cotton smoke were delivered.

Immediately after the injury, anesthesia was discontinued and the animals were allowed to awaken and were extubated. Animals were resuscitated by intraperitoneal injection of 0.9% saline (100 ml/kg) immediately after injury, followed by an additional injection of saline (50 ml/kg) every 24 h during the remainder of the study period. Buprenorphine (2 mg/kg) was subcutaneously administered for analgesia in all animals every 24 h. The animals were allowed to awaken and were extubated. Animals were resuscitated by intraperitoneal injection of 0.9% saline (100 ml/kg) immediately after injury, followed by an additional injection of saline (50 ml/kg) every 24 h during the remainder of the study period. Buprenorphine (2 mg/kg) was subcutaneously administered for analgesia in all animals every 24 h. The animals were then returned to their cages and allowed free access to food and water.

**Study criteria and variables.** Mice that were alive at the various time points (preinjury, 3 h, 6 h, 12 h, and 24 h) and not moribund (identified as having ruffled fur, unresponsive to noise, or comatose) were randomly selected for study. These mice were then anesthetized with isoflurane anesthesia, and samples of tissues were obtained from them. Twenty-four animals were used for each of the following variables: histology, wet/dry, myeloperoxidase (MPO) activity, NOS activity, RT-PCR.

**Mortality study.** Mice in all groups (n = 10) were observed every 12 h for 120 h after the injury. No other parameters were measured in these mice.

**Pulmonary gas exchange.** Mice were killed under anesthesia at various time points (0, 12, 24, 36, and 48 h) after the injury, and blood samples for blood gas analysis (blood gas analyzer Premier 300, Instrumental Laboratory, Lexington, MA) were obtained by direct withdrawal from the left ventricle.

**Lung histopathology.** Lung tissues were taken 48 h after injury for histological analysis. The lungs were infused via the airway with 1.4 ml of 10% phosphate-buffered formalin at 25-cm fluid height, embedded in paraffin, sectioned (4 µm), and stained with hematoxylin and eosin (15). A pathologist who was unaware of the group assignment analyzed the samples and scored the histological changes. Twenty-four areas of the lung parenchyma were graded on a scale of 0–4 (0, absent and appears normal; 1, light; 2, moderate; 3, strong; 4, intense) for congestion, hemorrhage, septal thickening, and edema. The mean score for each of the parameters was calculated, and then a total histopathological score (maximum: 16) was derived from the sum of the mean scores of the four variables.

**Measurement of lung wet-to-dry weight ratio.** Mice were killed under isoflurane anesthesia at various time points after injury, and lung tissues were taken for measurement of lung wet-to-dry weight ratio, which is an estimate of the degree of lung water content (19). The lung tissues were weighed and then dried to a constant weight in an oven at 65°C for 2 days (31). The wet-to-dry weight ratio was obtained by dividing the wet weight by the final weight of the dried lungs.

**Assay of lung MPO activity.** Quantitation of neutrophils in the lung was evaluated by measuring lung MPO activity as described previously (17, 25). Mice were killed at various time points after injury. The lungs were removed, weighed, homogenized (Physcion; Niti-on, Tokyo, Japan) in 10% (wt/vol) 0.05 mol/l phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide, and sonicated for 20 s. After centrifugation (4,500 g for 20 min at 4°C), 0.1 ml of the supernatant was added to 0.55 ml of 0.1 M phosphate buffer (pH 6.0) containing 1.25 mg/ml o-dianisidine and 0.05% hydrogen peroxide. After 5 min, changes in absorbance at 460 nm were measured with a spectrophotometer. The activity of purified known human neutrophil MPO was used as the standard. Results are expressed as units of MPO activity per gram of tissue.

**Measurement of plasma levels of NOs.** The NO levels in mice were evaluated by measuring the plasma concentration of the...
intermediate and end products, NO$_3^-$ and NO$_2^-$, as described previously (5). For conversion of nitrate to nitrite, the plasma samples were mixed with vanadium(III) and hydrochloric acid at 90°C in the NO$_2^-/NO_3^-$ reduction assembly (ANTEK model 745, Antec Instruments, Houston, TX). Thereafter, the NO reacted with ozone in the reaction chamber of the chemiluminescent NO detector (ANTEK model 7020, Antec Instruments), and the emitted light signal was recorded by dedicated software as the NO content (µmol/l).

**Measurement of NOS activity in lung tissue.** Lung tissue NOS activity was evaluated by conversion of L-[³H]arginine to L-[³H]citrulline with a NOS activity assay kit according to the manufacturer’s instructions (Cayman Chemical, Ann Arbor, MI).

**RNA isolation and analysis of iNOS mRNA by semiquantitative reverse transcription polymerase chain reaction.** Total RNA was isolated from lungs by the method of Chomczynski and Sacchi (3) with a commercial reagent (Ultraspec RNA, Biotecx Laboratories, Houston, TX). The first-strand cDNA was synthesized from the RNA as described previously (21).

The cDNA was used as template for polymerase chain reaction (PCR) amplification of iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cycling conditions were 94°C for 45 s, 55°C for 45 s, and 72°C for 90 s, as previously described (18). The primer sequences for iNOS were 5'-GTGGTGACAAGCACATT-TGG-3' (sense) and 5'-GGCTGGACTTTTCACTCTGC-3' (antisense). The primer sequences for GAPDH were 5'-GTGAAGGTCG-GTGTGAACGGATT-3' (sense) and 5'-TTATATGGGGTCT-GGATGGAA-3' (antisense). The plateau phase of iNOS and GAPDH PCR became apparent after 35 and 25 cycles, respectively. Thus we used 32 cycles for iNOS and 22 cycles for GAPDH. The PCR products were fractionated with electrophoresis on a 1.2% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. Densitometric analysis was performed with the public domain Image J program from NIH, which is available at http://rsf.info.nih.gov/nih-image. The iNOS PCR was standardized with the GAPDH PCR from the same RNA samples.

**Statistics.** Data are presented as means ± SE. Survival within 120 h was analyzed by the Cox-Mantel test. Other results were compared through a nonparametric Kruskal-Wallis test. A value of P < 0.05 was considered significant.

**RESULTS**

**Survival rate and pulmonary gas exchange.** All mice in the sham injury group survived at 120 h after isoflurane anesthesia (Fig. 1). The survival rate was 70% in mice subjected to burn injury and 60% in mice subjected to smoke inhalation injury alone at 120 h after the injury (Fig. 1A). The survival rate was 30% in mice subjected to combined burn and smoke inhalation injury at 120 h after the injury, a rate Fig. 2. Lung histopathological examination 48 h after burn injury alone, smoke inhalation injury alone, or combined burn and smoke inhalation injury. A–D: histopathological findings in lungs stained with hematoxylin and eosin (original magnification ×200) in sham injury group (A), burn injury group (B), smoke inhalation group (C), and combined burn and smoke inhalation group (D). E: total histology score in congestion, hemorrhage, septal thickening, and edema was calculated as an index of lung injury. Data are mean ± SE values for 6 animals. *P < 0.05 vs. sham injury group; †P < 0.05 vs. burn injury group; §P < 0.05 vs. smoke inhalation injury group.
significantly reduced compared with the other three groups (Fig. 1A).

Pulmonary gas exchange was evaluated by measuring PaO₂-to-FIO₂ ratio. The latter was significantly decreased in all injured groups (burn alone, smoke alone, and combined burn and smoke) compared with the sham injury group (Fig. 1B). However, PaO₂/FIO₂ was depleted more severely in mice subjected to combined burn and smoke inhalation, reaching a level below 200 at 48 h after injury.

**Lung histological changes.** At 48 h after burn injury alone, smoke inhalation injury alone, or combined burn and smoke inhalation injury, congestion, hemorrhage, septal thickening, and edema were observed in histological sections of lung (Fig. 2, B–D). Figure 2E shows quantitatively analyzed histopathological changes of lung tissue for congestion, hemorrhage, septal thickening, and edema. The total histology score was calculated as an index to evaluate overall tissue injury. Burn injury alone, smoke inhalation injury alone, and combined burn and smoke inhalation injury significantly increased (P < 0.0001) the total scores compared with sham injury (Fig. 2E). The combined injury showed higher scores compared with either burn or smoke inhalation injury alone (Fig. 2E).

**Changes in lung wet-to-dry weight ratio.** Lung wet-to-dry weight ratio was significantly higher (P < 0.0001) in mice subjected to combined burn and smoke inhalation injury than in mice that had a sham operation at 6, 12, 24, and 48 h after the injury (Fig. 3A). Twenty-four hours after burn injury alone, smoke inhalation injury alone, and the combined injury all mice showed significantly increased ratios (P = 0.0014, P = 0.0203, P < 0.0001, respectively) compared with sham-operated mice (Fig. 3B). The combined injury showed a significant increase compared with burn injury alone or smoke inhalation injury alone (P < 0.0001 and P = 0.0002, respectively) (Fig. 3B).

**Changes in lung MPO activity.** Lung tissue levels of MPO activity were increased 6 h after combined burn and smoke inhalation injury, reaching a maximum level at 12 h after the combined injury and gradually decreasing thereafter (Fig. 4A). Twelve hours after injury, the levels of MPO activity in the combined injury group significantly increased compared with the burn injury alone and smoke inhalation injury alone groups (Fig. 4B).

**Changes in plasma levels of NO₂/NO₃.** Three hours after combined burn and smoke inhalation, plasma levels of NO₂/NO₃ were significantly increased compared with pre-injury levels, and the levels peaked at 6 h after the injury (Fig. 5A). At 6 h after injury, all injured groups (burn injury alone, smoke inhalation injury alone, and combined burn and smoke inhalation injury) showed statistically increased

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**Fig. 3.** Changes in lung wet-to-dry weight ratio after burn and smoke inhalation injury and effects of burn injury alone, smoke inhalation injury alone, and combined burn and smoke inhalation injury. A: lung wet-to-dry weight ratios were evaluated just before injury (Pre) and 3, 6, 12, 24, and 48 h after injury. Data are mean ± SE values for 6 animals. †P < 0.05 vs. preinjury; ‡P < 0.05 vs. sham injury group. B: lung wet-to-dry weight ratios were determined at 24 h after injury. Data are mean ± SE values for 6 animals. *P < 0.05 vs. sham injury group; †P < 0.05 vs. burn injury group; ‡P < 0.05 vs. smoke inhalation injury group.

**Fig. 4.** Changes in lung myeloperoxidase (MPO) activity in mice subjected to combined burn and smoke inhalation injury and effects of burn injury alone, smoke inhalation injury alone, and combined burn and smoke inhalation injury. A: lung tissue levels of MPO activity were evaluated just before injury (Pre) and 3, 6, 12, and 24 h after injury. Data are mean ± SE values for 6 animals. †P < 0.05 vs. preinjury; ‡P < 0.05 vs. sham injury group. B: lung levels of MPO activity were determined at 12 h after the injury. Data are mean ± SE values for 6 animals. *P < 0.05 vs. sham injury group; †P < 0.05 vs. burn injury group; ‡P < 0.05 vs. smoke inhalation injury group.
pathology of ALI is a syndrome of inflammation and increased vascular permeability in the absence of left heart failure (13). In the present study, we introduced a mouse model of ALI and demonstrated a mechanistic role of iNOS-generated NO in burn injury, smoke inhalation injury, and combined burn and smoke inhalation injury. By inserting a custom-made endotracheal tube into the trachea and using a smoker device designed and constructed in our laboratory, we were assured that 1) the intubation process reduced the possibility of laryngospasm during the smoke administration process; 2) smoke administration was the primary cause of lung injury; and 3) the delivery of cooled smoke directly into the trachea (and then into the lungs) ensured that the ensuing pathophysiological changes were attributed to a response to irritants present in the smoke.

We found that mortality increased significantly in mice that suffered combined burn and smoke inhalation injury compared with burn injury and smoke inhalation injury alone. The mice subjected to combined burn and smoke inhalation injury also developed severe signs of deteriorated pulmonary gas exchange. The PaO2-to-FIO2 ratio in these mice was below 200 at 48 h after injury, which is consistent with data obtained from our ovine model of ALI (9). Additionally, lung histological analysis demonstrated more alveolar septal thickening and airway congestion in the combined burn and smoke injury group. Widespread obstruction of airways is a severe problem in smoke inhalation. The obstructing material in the ovine model is known to be a solid cast and is composed of sloughed bronchial epithelial
Interestingly, iNOS mRNA levels reached a maximum at 6 h after combined burn and smoke inhalation injury and showed a decrease 6 h later (Fig. 7). This observation suggests that burn and smoke inhalation injury, in addition to increasing the host’s immune response via iNOS expression and activity, will over time express inhibitors to this molecule’s activity (decreased plasma concentration of NO$_2$/$\text{NO}_3^-$) and decrease gene expression. A recent study by Kelleher et al. (10) identifies iNOS-derived NO as being able to negatively regulate DNA binding of NF-$\kappa$B, and therefore attenuate its activity.

Activated neutrophils have been implicated as being a major effector of cell damage and lung injury (26, 1). Once activated, polymorphonuclear neutrophils release MPO, an enzyme that catalyzes the formation of hyperchlorous acid from chloride ions and hydrogen peroxide (11). MPO levels in mice that received combined burn and smoke inhalation injury were significantly higher than in mice that received no injury, burn injury alone, or smoke inhalation injury alone, indicating the presence of a larger number of activated neutrophils. Clinically, patients suffering from ALI/ARDS have a dramatic increase in pulmonary neutrophils, as these are sequestered within the lung (13, 33). It has been demonstrated that because of the difference in caliber the larger neutrophils must deform and elongate to enter the smaller capillaries in the pulmonary bed (20). In response to stimuli, however, the submembranous cytoskeleton F-actin is formed and increases in number, leaving the neutrophils stiff and unable to deform (20). Consequently, these stiffened cells become trapped within the narrow pulmonary microvasculature (4).

The process by which excess NO produces an increase in vascular permeability is still unknown. However, hydroxyl radicals released by activated neutrophils have been shown to cause lipid peroxidation of the membranous bilayer and create endothelial cell damage, leading to increased microvascular permeability (2, 27). Neutrophils, once activated, shed their L-selectin on the cell surface and express P-selectin glycoprotein ligand-1, which binds to P-selectin on the surface of the endothelial cells and platelets (8, 7). Activated neutrophils produce reactive oxygen species that combine with NO released from pulmonary epithelial cells and macrophages to produce peroxynitrite and other reactive nitrogen species, which in turn produce singlestrand breaks in DNA (12). In future studies, we would like to test whether these findings hold true in our murine model.

Our experimental design included very strict animal exclusion criteria so that the data presented reflect mice that were not moribund. However, a possible limitation in our study is the absence of blood gas measurements to confirm smoke-related injury. Nevertheless, we demonstrated in the present study the time changes of inflammatory indexes such as excessive NO production and neutrophil activation in response to cutaneous burn and smoke inhalation. We observed a strong relationship between the combined injury and augmented lung damage as well as lung edema formation. Furthermore, we observed a more pronounced accumulation of neutrophils as well as elevated levels of iNOS gene transcription and activity in the lung tissue in mice exposed to the combined injury. The availability of different antibodies and genetically modified mice enhances the significance of the murine model of burn and smoke inhalation.
injury. We believe that our novel murine model will help investigators to explore various mechanistic aspects underlying cardiopulmonary morbidity secondary to burn trauma with or without concomitant smoke inhalation injury.

REFERENCES


