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THE 5-LIPOXYGENASE PATHWAY IS REQUIRED FOR ACUTE LUNG INJURY FOLLOWING HEMORRHAGIC SHOCK

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Abstract

The cellular and biochemical mechanisms leading to acute lung injury and subsequent multiple organ failure are only partially understood. In order to study the potential role of eicosanoids, particularly leukotrienes, as possible mediators of acute lung injury, we used a murine experimental model of acute lung injury induced by hemorrhagic shock after blood removal via cardiac puncture. Neutrophil sequestration as shown by immunofluorescence, and protein leakage into the alveolar space, were measured as markers of injury. We used liquid chromatography coupled to tandem mass spectrometry to unequivocally identify several eicosanoids in the bronchoalveolar lavage fluid of experimental animals. MK886, a specific inhibitor of the 5-lipoxygenase pathway, as well as transgenic mice deficient in 5-lipoxygenase, were used to determine the role of this enzymatic pathway in this model. Leukotriene B₄ and leukotriene C₄ were consistently elevated in shock-treated mice compared to sham-treated mice. MK886 attenuated neutrophil infiltration and protein extravasation induced by hemorrhagic shock. 5-lipoxygenase-deficient mice showed reduced neutrophil infiltration and protein extravasation after shock treatment, indicating greatly reduced lung injury. These results support the hypothesis that 5-lipoxygenase, most likely through the generation of leukotrienes, plays an important role in the pathogenesis of acute lung injury induced by hemorrhagic shock in mice. This pathway could represent a new target for pharmacological intervention to reduce lung damage following severe primary injury.

Keywords

multiple organ failure; eicosanoids; leukotrienes; mass spectrometry; inflammation; pharmacological inhibition; transgenic mice

INTRODUCTION

Acute lung injury (ALI) with subsequent multiple organ failure (MOF) is the leading cause of delayed mortality following traumatic injury, and it consumes enormous health care resources. Although splanchnic ischemia has been implicated in the pathogenesis of ALI, the mechanism remains unclear (1–3). Our prior work suggested a link between increased type IIa secretory phospholipase A₂ activity and MOF in severely injured patients (4), and

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our investigation with trauma/hemorrhagic shock suggested a key role for phospholipase A₂-dependent lipids in the development of ALI mediated by neutrophils (PMN) (1,5,6). Different lipid mediators emanate from free arachidonic acid liberated by phospholipase A₂, the most prominent being prostaglandins, thromboxanes, and prostacyclin derived from the action of cyclooxygenase (COX), and leukotrienes derived from the action of 5-lipoxygenase (5-LO) (7,8). Although COX products have been studied extensively in large clinical trials of sepsis-induced lung dysfunction employing NSAIDs as inhibitors, there has been a paucity of data to suggest a benefit (9,10). On the other hand, evidence has been gathered to implicate leukotrienes in experimental lung injury following diverse insults (11), and in clinical investigations of lung disease such as asthma (12).

Leukotrienes are believed to be critical in the recruitment and activation of PMN as well as the induction of pulmonary edema (12–15). Leukotrienes are produced by the metabolism of arachidonic acid by 5-LO and its cofactor 5-lipoxygenase activating protein (FLAP), which yield leukotriene A₄ (LTA₄). LTA₄ is then acted on by either LTA₄ hydrolase or LTC₄ synthase to produce leukotriene B₄ (LTB₄) or leukotriene C₄ (LTC₄), respectively (8). LTB₄ is involved in PMN chemotaxis and adherence to endothelial cells, and stimulates the release of superoxide radicals from PMNs (14). Elevated LTB₄ levels have been observed in several inflammatory states including inflammatory bowel disease and acute respiratory distress syndrome (ARDS) (16–18). LTC₄ and its metabolites LTD₄ and LTE₄, collectively known as cysteinyl leukotrienes, are involved in vascular constriction and edema formation (19), which are strongly implicated in asthma and ALI (20).

We hypothesized that products of arachidonate oxidation were critical to the development of hemorrhagic shock-induced lung injury. As a first step we investigated whether any arachidonate products could be found in the lung lavage. Based on the lipid mediators observed, we induced hemorrhagic shock in animals treated with an inhibitor of leukotriene production, and in transgenic animals genetically deficient in 5-lipoxygenase. Our data indicated a critical role of 5-lipoxygenase in the pathogenesis of post-hemorrhagic shock ALI.

METHODS

Animals

C57BL/6 wild type and 5-lipoxygenase $-/-$ mice, 8 to 12 week-old, were from The Jackson Laboratory (Bar Harbor, ME). All experiments were approved by the University of Colorado Denver Institutional Animal Care and Use Committee, and conformed to the *Guide for the Care and Use of Laboratory Animals* (National Research Council, revised 1996).

Hemorrhagic shock model

The mouse hemorrhagic shock model has been described previously (21). Briefly, mice were anesthetized by i.p. administration of 80 mg/kg ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 12 mg/kg xylazine (VEDCO, St. Joseph, MO), and shock was induced by removing 30% of the calculated blood volume via cardiac puncture. The sham procedure involved cardiac puncture but no blood removal. No evidence of bleeding into the pericardial space or hemothorax was found, and there were no signs of lung or cardiac contusion. In some experiments, a group of wild type animals received an injection of 6 mg/kg of the FLAP inhibitor MK886 (Cayman Chemical Company, Ann Arbor, MI), or of vehicle (DMSO), via tail vein injection 30 min prior to the induction of hemorrhagic shock. After HS induction and a 4-h recovery period under a heat lamp with free access to food and water, animals were euthanized. Bronchoalveolar lavage was performed (three times) with 1

mL saline via tracheotomy. Typical recovery was 60–75% of the volume. Bronchoalveolar lavage fluid (BALF; 200 μ L) was mixed with 2 vol methanol and stored at -80°C before lipid extraction. Additional aliquots were used for protein assay. The pulmonary vasculature was rinsed with 10 mL PBS through the right ventricle. After clamping the left main stem bronchus, the left lung was removed and used for myeloperoxidase assay. In some experiments, part of the left lung was cut, weighed, placed in lysis buffer (complete Lysis-M, EDTA-free, Roche Applied Science, Indianapolis, IN), homogenized and mixed with 2 vol methanol for mass spectrometry analysis of eicosanoids. The right lung was inflated with optimal cutting temperature medium, frozen in dry ice-chilled isopentane and used for immunofluorescence analysis.

Lung PMN accumulation was determined by immunofluorescence and myeloperoxidase activity as previously described (21). For immunofluorescence, tissue cryosections (5 μm -thick) were collected on poly-L-lysine-coated slides, treated with a mixture of acetone/methanol 7:3 (v/v) for 10 min, washed with PBS and fixed with 4% paraformaldehyde for 10 min. Sections were washed, blocked with 10% normal donkey serum for 30 min and incubated overnight at 4°C with a monoclonal rat antibody against the p40 neutrophil antigen (Ly-6B.2), a GPI-anchored protein expressed in neutrophils but not in resident macrophages (clone 7/4; AbD-Serotec, Kidlington, UK; 1:500 dilution in PBS containing 1% BSA). Sections were then washed and incubated with Cy3-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA; 1:150 dilution in PBS/BSA). The cell surface was counterstained with fluorescein-conjugated wheat germ agglutinin (Invitrogen, Carlsbad, CA; 1:1000 dilution in PBS/BSA), and nuclei were counterstained with Hoechst 33342 (Invitrogen; 1:33 dilution in PBS/BSA) for 45 min. Sections were then washed, mounted and sealed. Microscopy analysis was performed with a Leica DMRXA digital microscope equipped with Slidebook software (Intelligent Imaging Innovations, Denver CO). Neutrophils in 5 random fields (800 x 800 pixels or 0.017 mm^2 /field) were counted by a viewer blinded to the experimental conditions.

As an indicator of lung injury (22,23), total protein in BALF was measured by a micro BCA protein assay kit (Thermo Scientific, Rockford, IL).

Eicosanoid analysis

Liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) analysis was performed on either an AB Sciex 5500 QTRAP mass spectrometer or an AB Sciex 3000 tandem quadrupole mass spectrometer. BALF eicosanoids were extracted and analyzed as described before (24), with some modifications as stated below. Internal standards (Cayman Chemical Company, Ann Arbor, MI) were added as follows: 500 pg each of $[\text{d}_4]\text{LTB}_4$, $[\text{d}_4]\text{PGE}_2$, $[\text{d}_4]\text{TXB}_2$, $[\text{d}_4]13\text{-HODE}$, $[\text{d}_8]5\text{-HETE}$ and 2.5 ng each of $[\text{d}_5]\text{LTC}_4$, $[\text{d}_4]6\text{-keto-PGF}_{1\alpha}$ and $[\text{d}_8]\text{AA}$. Solid phase cartridges were Strata-X, 33 μm Polymeric Reversed Phase (Phenomenex, Torrance, CA). Chromatography was performed on a Kinetex C18, 50 x 3 mm, 2.6 μm column (Phenomenex), at 200 $\mu\text{L}/\text{min}$ with a linear gradient from 40% solvent B (methanol/acetonitrile 35:65, v/v) to 75% starting at 1 min to 6 min, 75% to 98% in 1 min, a 6 min hold and re-equilibration for 8 min (solvent A is 8.3 mM ammonium acetate, pH 5.7). Multiple reaction monitoring was performed using the following m/z transitions (parent ion $\rightarrow$ product ion): 335 $\rightarrow$ 195, LTB_4 and $\Delta^6\text{-trans-LTB}_4$ s; 351 $\rightarrow$ 195, 20-OH- LTB_4 ; 365 $\rightarrow$ 195, 20-COOH- LTB_4 ; 624 $\rightarrow$ 272, LTC_4 ; 495 $\rightarrow$ 177, LTD_4 ; 438 $\rightarrow$ 333, LTE_4 ; 319 $\rightarrow$ 115, 5-HETE; 319 $\rightarrow$ 179, 12-HETE; 319 $\rightarrow$ 219, 15-HETE; 303 $\rightarrow$ 259, AA; 295 $\rightarrow$ 171, 9-HODE; 295 $\rightarrow$ 195, 13-HODE; 351 $\rightarrow$ 271, PGE_2 and PGD_2 ; 353 $\rightarrow$ 309, $\text{PGF}_{2\alpha}$; 369 $\rightarrow$ 163, 6-keto- $\text{PGF}_{1\alpha}$; 369 $\rightarrow$ 169, TXB_2 ; 629 $\rightarrow$ 272, $[\text{d}_5]\text{LTC}_4$; 327 $\rightarrow$ 116, $[\text{d}_8]5\text{-HETE}$; 339 $\rightarrow$ 197, $[\text{d}_4]\text{LTB}_4$; 299 $\rightarrow$ 198, $[\text{d}_4]13\text{-HODE}$; 311 $\rightarrow$ 267, $[\text{d}_8]\text{AA}$; 373 $\rightarrow$ 173, $[\text{d}_4]\text{TXB}_2$; and 355 $\rightarrow$ 275, $[\text{d}_4]\text{PGE}_2$.

Statistical Analysis

Results were analyzed by ANOVA tests with post-hoc Bonferroni correction using Statview 5.0 (SAS Institute, Cary, NC), and were expressed as average $\pm$ SEM. The number of animals in each group was: sham, n=4; heat-shock (HS), n=4; vehicle (DMSO), n=3; MK886, n=5; 5-LO $-/-$, n=7.

RESULTS

Hemorrhagic shock induces neutrophil recruitment and acute lung injury

In this study we used a known murine model of hemorrhagic shock induced by blood removal via cardiac puncture. A key cellular mediator of the development of ALI and subsequent MOF is the neutrophil (6,20,25,26); therefore the measurement of PMN recruitment in the lung was used as verification of hemorrhagic shock-induced lung injury. Histological lung sections were stained with an antibody specific for mouse PMNs (Figure 1A). Shocked wild type mice clearly showed an increase in PMN infiltration compared to sham-operated animals (Figure 1A, top panels). When quantitated, shocked mice showed a 9-fold increase in PMN per high-power field (Figure 1B).

Identification of eicosanoids in bronchoalveolar fluid following hemorrhagic shock

Increased levels of pro-inflammatory molecules in the BALF of humans with ALI and its more severe manifestation, ARDS, have been well described in the clinical literature (18,20,27). In order to elucidate the factors responsible for the development of ALI following hemorrhagic shock in the mouse, we utilized LC/MS/MS to screen for eicosanoids in the BALF.

Identification of eicosanoids was carried out using the chromatographic behavior as well as mass spectrometric multiple reaction monitoring, where specific product ions were derived from the molecular anions of the eicosanoids. The most abundant eicosanoids in the BALF extracts from hemorrhagic shock animals were found to be metabolites of 5-LO including LTB₄, LTC₄, and LTE₄ (Fig. 2). None of these eicosanoids was found in sham-operated mice, except for a very small amount of LTE₄ in some of these animals (Fig. 2, left panels), as judged by the specific *m/z* transition and elution at the times indicated by the stable isotope-labeled internal standards. However, in the BALF samples from shock animals (Fig. 2, right panels), there was clear evidence for the presence of LTB₄, LTC₄, and LTE₄. This experiment was performed multiple times and although there was variability in the absolute amounts of leukotrienes in the BALF of hemorrhagic shock animals, they were observed in every sample in this group. LTC₄, and to a lesser extent LTB₄ and LTE₄, were also detected in the lungs of these animals (data not shown).

Metabolites of the cyclooxygenase pathway were observed in some but not all BALF samples of animals with hemorrhagic shock, including 6-keto-PGF_{1 α} , PGE₂, and TXB₂. The results from the analysis of a set of sham and shock animals are presented (Figure 3) as a typical example. In these animals, very low levels of PGE₂ did appear in the BALF from both control and HS-treated animals (Figure 3, top panels), somewhat more abundant in the latter. For this set of animals there was no evidence of TXB₂, but in other experimental sets there was some TXB₂ in both the sham and HS BALF (data not shown). Overall, there was no striking evidence for induction of COX products appearing in BALF as a consequence of hemorrhagic shock.

A low level of 5-HETE was observed in the BALF of injured animals (Fig. 3), and there was some evidence for the production of other lipoxygenase products. Monohydroxylated eicosanoids were observed, including 15-HETE and 5-HETE (Figure 3, bottom panel) both

of which could be derived from the action of 15- and 5-lipoxygenase respectively. Detection of the 15-HETE and 12-HETE (data not shown) products was variable in all animals studied, with no clear trend about relationship to hemorrhagic shock. Overall, the 5-lipoxygenase products were consistently and unambiguously observed in BALF of mice after hemorrhagic shock.

Role of the 5-lipoxygenase pathway in hemorrhagic shock-induced neutrophil recruitment

Analysis of the BALF following hemorrhagic shock in mice suggested a role for 5-LO in the development of lung injury via elevations in LTB₄ and LTC₄ levels. To determine if 5-LO plays a functional role in the development of post-shock ALI, we used both a pharmacological approach and a genetic approach with transgenic mice.

In one experimental group, shock was induced after the i.v. administration of the FLAP inhibitor MK886, which has been shown to inhibit leukotriene production *in vivo* (8,28,29). Mice treated with MK886 had reduced neutrophil infiltration as observed by immunofluorescence (Fig. 1A). Neutrophil infiltration was reduced nearly 50% (Fig. 1B). PMN recruitment did not return to sham levels (Fig. 4), but leukotriene production was undetectable at this dose of MK886 as determined by our analysis. Combined, these data suggest that while products of the 5-LO pathway are involved in PMN recruitment, they are not the only factors involved.

In order to independently establish a role for 5-LO in the development of ALI, 5-LO-deficient mice were subjected to hemorrhagic shock, and PMN accumulation in the lung was studied. PMN recruitment was attenuated by about 50% in the 5-LO $-/-$ group (Fig. 1B). No leukotrienes were detected in these mice (data not shown). As in MK886-treated mice, PMN recruitment was only partially inhibited, suggesting that additional mechanisms other than leukotrienes are involved in this process.

Role of 5-lipoxygenase in hemorrhagic shock-induced acute lung injury

A widely used standard to evaluate the level of ALI in animal models has been the measurement of BALF protein levels (22,23,30,31). Therefore, we determined protein concentration in the BALF collected from mice subjected to this model of hemorrhagic shock (Fig. 5). Shocked mice were found to have a 3.1-fold increase in the level of BALF total protein compared to sham animals. The increases in BALF protein concentration, together with PMN recruitment data, supported this as a clinically relevant model for ALI. In MK886-treated mice and in 5-LO $-/-$ animals, the levels of protein were nearly identical to those in sham-operated animals. This indicated that 5-LO activity plays an essential role in the etiology of hemorrhagic shock-induced lung injury.

DISCUSSION

The results of this study reveal an important role played by products of the 5-lipoxygenase enzymatic pathway in the development of acute lung injury as a consequence of hemorrhagic shock. Several animal models of hemorrhagic shock have been developed to replicate what is seen clinically in human patients (5,21,32,33). Our murine model involves induction of hemorrhagic shock via cardiac puncture (21,33,34). This model was chosen because of prior studies demonstrating its reproducibility and the fact that it limits tissue injury, which has been shown in turn to cause additional injury-related changes (21,32,35,36).

One key contribution of this work is strong evidence for the presence of LTB₄, LTC₄, LTE₄ and 5-HETE, products of the 5-LO pathway, specifically in the airways of hemorrhagic shock-treated mice. In contrast, there was no clear evidence for the unique appearance of

products of the cyclooxygenase pathway of arachidonate metabolism. Leukotrienes have been associated with several chronic inflammatory diseases such as asthma, allergic rhinitis, inflammatory bowel disease, rheumatoid arthritis, and psoriasis. LTB₄ has been found to be elevated in patients with ARDS (17,18), and it probably plays a role in lung injury through its ability to attract and activate PMNs (12–15,28,29). The PMN has been recognized as the critical inflammatory cell in the pathogenesis of ALI. Trauma and subsequent hemorrhagic shock have been shown to increase not only pulmonary PMN recruitment, but also the oxidative potential of the recruited cells (6,25). On the other hand, cysteinyl leukotrienes, like LTC₄, are known to be involved in lung injury in humans, most notably in asthma, probably through some of their known biological actions on lung epithelial cells (mucus secretion), smooth muscle cells (bronchoconstriction) and vascular endothelial cells (increased vascular permeability).

The observation suggests that leukotrienes are made by cells resident in or recruited to the lung during the injury process, since it is widely accepted that leukotrienes exert their actions in an autocrine or paracrine fashion, and they are rapidly metabolized and cleared from the circulation (37). Neutrophils, which are known to play an important role in this model and are known to be sequestered in the lung microvasculature, could likely be the source for LTB₄. However, they do not express LTC₄ synthase, therefore the source of LTC₄ could be alveolar macrophages, mast cells or monocytes recruited subsequently to PMN infiltration, a common occurrence in inflammatory responses. It is also possible that PMN may provide LTA₄ to endothelial cells or platelets in the vasculature, and that LTC₄ may be synthesized by these cells in an instance of transcellular metabolism (38). The sequence of events is not totally understood, but HS probably triggers a local response in the lungs through chemical mediators such as cytokines and through cellular mediators such as neutrophils being sequestered in the lung microvasculature. This effect is probably not limited to hemorrhagic shock, as other experimental models of ischemia also result in lung injury which depends on neutrophils and eicosanoids (39).

Of major concern was whether the production of these mediators in the hemorrhagic shock model played a role in the progression of lung damage or they were merely produced as a result of injury. The potential mechanistic role of 5-LO in the pathogenesis of ALI was examined by inhibiting the first enzymatic step in the oxidation of arachidonic acid leading to leukotriene synthesis, both through pharmacological blockade with the FLAP inhibitor MK886 and by using mice deficient in 5-LO. Both approaches strongly supported the hypothesis that the 5-LO pathway plays a major role in mediating lung injury in this model, since MK886 treatment and 5-LO deficiency result in reduced neutrophil recruitment and reduced protein levels in the bronchoalveolar lavage fluid of treated mice.

It is interesting to note that neither the 5-LO ^{-/-} nor the MK886 group had a return of PMN recruitment/sequestration to control levels (sham treated), suggesting that leukotrienes have an important role in hemorrhagic shock-induced PMN recruitment, but that other mediators clearly contribute to this complex response in the lung. While LTB₄ is a known powerful PMN chemoattractant, other compounds such as granulocyte colony-stimulating factor (G-CSF), formyl-methionyl-leucyl-phenylalanine (fMLP), complement factors, IL-8 or other cytokines may also play a role in PMN chemotaxis. In contrast to this effect on PMN recruitment, and as determined by the BALF protein levels, ALI was completely abrogated in both the 5-LO ^{-/-} group and the MK886 group after the induction of hemorrhagic shock. These data suggest that while several factors independent of 5-LO are involved in the recruitment of PMNs, the products of the 5-LO pathway act via a mechanism beyond other PMN chemoattractants to produce ALI. Whether that is by acting on additional target cells (such as the endothelium or the pulmonary epithelium) warrants further investigation.

The fact that pharmacological inhibition of the 5-LO pathway results in reduced symptoms in hemorrhagic shock-induced lung injury points at the possibility that this pathway could become a target in the management of clinical situations that could lead to lung injury and subsequent multiple organ failure. This involvement of the 5-LO pathway has been previously reported in a different model of MOF, induced by the intraperitoneal injection of zymosan in mice (40). In that study, 5-LO-deficient mice were protected from some of the effects of zymosan, as revealed by markers of lung, renal and pancreatic injury, although the presence of 5-LO products was not evaluated. This suggests that the involvement of 5-LO in mediating MOF could possibly occur in different types of injury.

In summary, we have found that 5-lipoxygenase and its products the leukotrienes play an important role in trauma/hemorrhagic shock-induced acute lung injury. With this understanding, it may be possible to pharmacologically target the effects of these proinflammatory eicosanoids by agents working either at their synthesis or on specific receptors in order to reduce the toll ALI and subsequent MOF has on individuals as well as our health care system.

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Figure 1. Hemorrhagic shock induces neutrophil recruitment in the lungs of mice

A) Immunofluorescence images of lung tissue from sham-operated or hemorrhagic shock-treated (HS) animals, either wild type (WT) or 5-LO-deficient. Plasma membranes are stained in green, nuclei in blue and neutrophils in purple (see Methods for details). Where stated, 6 mg/kg MK886 (Cayman Chemical Company, Ann Arbor, MI) was administered via tail vein injection 30 min before cardiac puncture. Images are representative of at least three experiments. (B) Quantitation of data from lung slices obtained from mice treated as indicated. Data (average $\pm$ SEM) are expressed as PMN number per high-power field (HPF). * $p < 0.05$ vs. sham-treated # $p < 0.05$ vs. HS-treated.

Figure 2. Leukotriene levels are increased in the bronchoalveolar fluid from hemorrhagic shock-treated mice

Multiple reaction monitoring (MRM) using ion transitions specific for LTB_4 (top panel), LTC_4 (middle panels) and LTE_4 (bottom panels) and stable isotope-labeled internal standards by LC/MS/MS. The insets in the top panels are for an expanded scale to view the lower signals for LTB_4 . All signals were normalized to the signal for each corresponding internal standard, which was added in an identical amount to each BALF sample before lipid extraction.

Figure 3. Cyclooxygenase and monohydroxyeicosanoid products are present in the bronchoalveolar fluid from control and hemorrhagic shock-treated mice
Multiple reaction monitoring (MRM) using ion transitions specific for PGE₂ (top panel), TXB₂ (middle panels) and HETEs (bottom panels) and indicated stable isotope-labeled internal standards by LC/MS/MS. All signals were normalized to the signal for each corresponding internal standard which was added in an identical amount to each BALF sample before extraction.

Figure 4. Myeloperoxidase activity in bronchoalveolar fluid from control and hemorrhagic shock-treated mice

BALF from wild type or knockout mice treated as indicated was analyzed for myeloperoxidase activity. MK886 was used at 6 mg/kg. HS, hemorrhagic shock. * $p < 0.05$ vs. sham-treated # $p < 0.05$ vs. HS-treated.

Figure 5. Protein concentration in bronchoalveolar fluid from control and hemorrhagic shock-treated mice

As a marker of lung injury, protein concentration was determined in BALF from wild type or knockout mice treated as indicated. MK886 was used at 6 mg/kg. HS, hemorrhagic shock. * $p < 0.05$ vs. sham-treated # $p < 0.05$ vs. HS-treated.