Published in final edited form as:

Ann Surg. 2018 February ; 267(2): 382–391. doi:10.1097/SLA.0000000000002070.

Molecular Characterization of Hypoxic Alveolar Epithelial Cells After Lung Contusion Indicates an Important Role for HIF-1 α

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Abstract

Objective—To understand the fate and regulation of hypoxic type II alveolar epithelial cells (AECs) after lung contusion (LC).

Background—LC due to thoracic trauma is a major risk factor for the development of acute respiratory distress syndrome. AECs have recently been implicated as a primary driver of inflammation in LC. The main pathological consequence of LC is hypoxia, and a key mediator of adaptation to hypoxia is hypoxia-inducible factor (HIF)-1. We have recently published that HIF-1 α is a major driver of acute inflammation after LC through type II AEC.

Methods—LC was induced in wild-type mice (C57BL/6), luciferase-based hypoxia reporter mice (*ODD-Luc*), and HIF-1 α conditional knockout mice. The degree of hypoxia was assessed using hypoxyprobe and in vivo imaging system. The fate of hypoxic AEC was evaluated by luciferase dual staining with caspases-3 and Ki-67, terminal deoxynucleotidyl transferase dUTP nick end labeling, and flow cytometry with ApoStat. NLRP-3 expression was determined by western blot. Laser capture microdissection was used to isolate AECs in vivo, and collected RNA was analyzed by Q-PCR for HIF-related pathways.

Results—Global hypoxia was present after LC, but hypoxic foci were not uniform. Hypoxic AECs preferentially undergo apoptosis. There were significant reductions in NLRP-3 in HIF-1 α conditional knockout mice. The expression of proteins involved in HIF-related pathways and inflammasome activation were significantly increased in hypoxic AECs.

Conclusions—These are the first in vivo data to identify, isolate, and characterize hypoxic AECs. HIF-1 α regulation through hypoxic AECs is critical to the initiation of acute inflammation after LC.

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Author contributions: Concept and design—MVS and KR; performed research—MAS, MVS, VAD, LKM, and XX; analysis and interpretation—MAS, MVS, LZ, DMA, YS, and KR; manuscript and contribution of important intellectual content—MAS, MVS, and KR.

The authors have nothing to disclose.

The authors report no conflicts of interest.

Keywords

alveolar epithelial cells; hypoxia; LCM; lung contusion

Lung contusion (LC) is caused by thoracic trauma, usually in the setting of motor vehicle accidents or blast injuries, and commonly requires hospitalization. Importantly, LC is a significant risk factor for the development of acute respiratory failure, most notably acute respiratory distress syndrome (ARDS) and secondary bacterial ventilator-associated pneumonia (VAP).^{1–3} The pathophysiology of and inflammatory response induced by LC are complex and multi-faceted, and for nearly a decade, our laboratory has investigated LC using a murine model of closed-chest blunt trauma.⁴

One of the main physiologic consequences of LC is hypoxia. This state of tissue oxygenation leads to the activation of a set of transcription factors called hypoxia-inducible factors (HIFs). HIFs subsequently mediate the cellular response to hypoxia by targeting hypoxia response elements (HREs) within the promoters of a subset of certain genes, such as those involved in metabolism, angiogenesis, and apoptosis.⁵ HIF-1 α is involved in the pathogenesis of injury and inflammation after trauma to the lung and elsewhere.^{6,7} Moreover, it has been recognized that HIF-1 α promotes apoptosis of alveolar epithelial cells in the setting of hypoxic injury.⁸

It was previously believed that alveolar epithelial cells (AECs) were not directly involved in the pathogenesis of lung injury. Type II AECs were indirectly implicated in the pathophysiology of LC in the context of surfactant dysfunction.^{4,9} Inflammatory cells such as macrophages and neutrophils were traditionally considered principally responsible for initiating pulmonary injury. In addition, inflammasomes—complex assemblages of sensor, adaptor, and effector proteins—are potent proinflammatory molecules involved in the immune response to a variety of infectious and sterile stimuli in the lung.^{10,11} It is more recently apparent that AECs are active players in the pulmonary immune response.¹² Our laboratory has recently demonstrated that type II AECs is involved in the inflammatory response to LC, specifically through activation of HIF-1 α .¹³ Additionally, we observed that HIF-1 α directly regulates interleukin (IL)-1 β , a key mediator of the acute inflammatory response after LC.^{14,15}

There are many factors, such as inflammation and hypoxia, that have the potential to stimulate and activate HIF-1 α -related pathways. However, the specific contribution of hypoxic versus normoxic AECs to the initiation of inflammation and subsequent physiological dysfunction after acute insults remains unknown. In the current study, we used hypoxia reporter mice to confirm and better characterize the fate of hypoxic AECs in the pathogenesis of LC. We hypothesized these hypoxic cells preferentially undergo apoptosis and drive the acute inflammatory response through a HIF-1 α -directed pathway. RNA samples from normoxic and hypoxic regions in oxygen-dependent domain luciferase (*ODD-Luc*) mice after LC were isolated via laser capture microdissection (LCM), and select endogenous hypoxia-related markers and inflammatory mediators were evaluated through Q-PCR. We believe this is the first report of hypoxic cells in the lungs that have been

isolated and characterized, and the results indicate that hypoxic activation of AECs through HIF-1 α is critical to the initiation of the inflammatory response after LC.

METHODS

Animals

Male age-matched (6–8 weeks) C57BL/6, ODD-Luc (Jackson Laboratories, Bar Harbor, ME), and HIF-1 α (triple transgenic conditional knockout specific for type II AEC, SP-C-rtTA_/tg/[(tetO) 7-CMV-Cretg/tg/HIF-1_ *flox/flox*]) mice were utilized in this study. The triple transgenic mice are capable of respiratory epithelium-specific conditional recombination in the floxed HIF-1 α gene upon exposure to doxycycline.¹³ All procedures performed were approved by the Institutional Animal Care and Use Committee at the University of Michigan and complied with state, federal, and National Institutes of Health regulations.

Murine Model For Lung Contusion

Wild-type (WT) (C57BL/6), ODD-Luc, and HIF-1 α mice weighing 20 to 25 g (age 6–8 weeks, bred inhouse) were anesthetized, and LC was subsequently induced.^{4,13,16}

Administration of Anesthetic, Analgesic, and Resuscitation

Animals were anesthetized by intraperitoneal (i.p.) injection of ketamine (80–120 mg/kg body weight) and xylazine (5–10 mg/kg body weight). Systemic analgesics were not used due to their effects on the immune/inflammatory system. In the event of severe respiratory distress beyond 48 hours, animals were humanely euthanized.

Murine Model of Gastric Aspiration

Mice were anesthetized with 5% isoflurine in oxygen at a rate of 5.l/min. After induction of anesthesia, mice were injected with 30 μ L of either normal saline, pH 5.3 (NS, vehicle control) or NS + HCl, pH 1.25 (ACID) via deep oral injection into the trachea. Animals were allowed to recover spontaneously.

IVIS of ODD-Luc Mice After Lung Contusion and Gastric Aspiration

The *ODD-Luc* mice after LC and gastric aspiration mice received a single i.p. injection of a mixture of luciferin (50 mg/ kg) in sterile water, and details are as previously described.¹³

Pimonidazole Administration (In Vivo) and Hypoxyprobe

Pimonidazole hydrochloride (Hypoxyprobe-1, Chemicon, and Temecula, CA) was administered at 60 mg/kg body weight in WT mice. This agent was dissolved in PBS and administered via the tail vein 1 hour before LC.¹⁷ Lung and liver samples were collected 24 hours after LC and then frozen in optimal cutting temperature (OCT) mounting medium (Sakura Finetek, Torrance, CA). For detection of pimonidazole, sections were incubated with fluorescein isothiocyanate (FITC) conjugated to mouse IgG1 monoclonal antibody (FITC-Mab1). A 1:100 dilution of secondary rabbit anti-FITC horseradish peroxidase

conjugated antibody in the Hypoxyprobe-1 Plus Kit was used (Hypoxyprobe, Inc, Burlington, MA).

Immunofluorescence Staining

Lung sections were prepared and stained as previously described.^{13,16}

Luciferase Staining—Frozen lung sections were exposed to a 1:50 dilution of primary anti-luciferase antibody (Abcam, Cambridge, MA) in PBST overnight. In caspase-3 staining, frozen lung sections were exposed to a primary rabbit anti-caspase-3 antibody (Abcam, Cambridge, MA) in PBST overnight. In Ki67 staining, frozen lung sections were exposed to a primary rabbit anti-Ki67 antibody (Abcam, Cambridge, MA) in PBST overnight. After washing, corresponding secondary antibody was used.

TUNEL Assay—Frozen lung sections were used in this study. The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) Apoptosis Detection Kit (EMD Millipore, Billerica, MA) was used according to the manufacturer's protocol for the detection of the endonucleolytic cleavage of chromatin that is characteristic of apoptosis. Finally, photomicrographs of the invasive sections were taken and analyzed digitally using Photoshop software version 9.0.2.

Luciferase Expression Assay

Dual luciferase reporter assay was performed according to the manufacturer's recommendations (Promega Corporation, Madison, WI).

Albumin Concentrations in Bronchoalveolar Lavage

Albumin concentrations in the BAL were measured by ELISA using polyclonal rabbit antimouse albumin antibody and HRP-labeled goat antirabbit IgG (Bethyl Laboratories, Inc., Montgomery, TX).^{13,16}

Determination of Cytokine Levels in BAL

Soluble concentrations of IL-6, IL-1 β , macrophage inflammatory protein 2 (MIP-2), CCL-12 [monocyte chemotactic protein 5 (MCP-5)], and KC in the BAL after LC were determined using ELISA as previously described.^{3,9,13,16,18}

ApoStat

Intracellular caspase activity was measured by Apostat (R&D Systems, Minneapolis, MN). BAL cells were washed and fixed with 1% formalin and reconstituted in 300 μ L of flow buffer.

Laser Capture Microdissection

Tissue sections were prepared by extracting fresh lung (no perfusion or fixation), placing sections in OCT-containing plastic containers to snap-freeze on dry ice, sectioning fresh-frozen tissue in cryostat (7 μ M thick), and dry mounting on superfrost plus slides. Tissue heterogeneity/cell targeting was visualized via immunostaining with antiluciferase antibody

to sections. The cells of interest (hypoxic and nonhypoxic) were identified and subsequently captured. This process was completed as indicated by the kit manufacturer (Arcturus Engineering, Mountain View, CA) for RNA extraction.¹⁹

TaqMan Quantitative Polymerase Chain Reaction

Total RNA was prepared from whole lung lysate as well cells obtained by LCM and was reverse-transcribed into cDNA using moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies Corporation, Grand Island, NY) as previously described.^{13,16}

Western Blot Analysis

Western blots were performed as previously described.¹³

Statistical Methods

Data were expressed as mean $\pm$ standard error of mean (SEM). Statistical significance was estimated using 1-way analysis of variance (Graph Pad Prism 6.01, La Jolla, CA). Individual intergroup comparisons were analyzed using 2-tailed, unpaired *t* test with Welch correction. Significance was set at *P* = 0.05.

RESULTS

ODD-Luc Mice as a Background Mouse Model for Study of Hypoxia After Lung Contusion and Gastric Acid Aspiration

Previous studies of LC and gastric acid aspiration (GA) have essentially been performed in C57BL/6 mice. *ODD-Luc* mice constitutively express a chimeric protein composed of the ODD of HIF-1 α fused to luciferase. HIF-1 α is ubiquitinated and degraded under normoxic conditions, but under hypoxia, the protein is active and in *ODD-Luc* mice produces readily measurable bioluminescence. We observed that after LC, there is significant bioluminescence in the lungs and abdominal organs of *ODD-Luc* mice, corresponding to hypoxia and associated injury (Fig. 1A). We next measured the degree of bioluminescence in the lung and liver of *ODD-Luc* mice after LC. Bioluminescence was significantly increased in the lung at 48 hours and in the liver at 24 and 48 hours after LC (Fig. 1B). Additionally, we found that after GA, there is significant bioluminescence in the lungs and abdominal organs (Fig. 2A and B). There was also increased bioluminescence in the explanted lung, liver, kidney, and spleen at 24 hours after GA (Fig. 2C). As a whole, these data endorse the use of *ODD-Luc* mice to study hypoxia in the setting of both focal and diffuse lung injuries, represented here as LC and GA, respectively.

ODD-Luc Mice Exhibit Increased Permeability Injury and Inflammation After LC

The injury and inflammatory response resulting from LC differ between mice of different genetic backgrounds. We therefore investigated the injury and inflammatory profile of *ODD-Luc* mice, which are of a friend virus strain B (FVB) background, after LC to assess whether it is similar to that observed in C57BL/6 WT mice. LC was induced in *ODD-Luc* mice. Bronchoalveolar lavage (BAL) was collected at 5, 24, 48, and 72 hours after insult, and also

from uninjured *ODD-Luc* mice. BAL was analyzed by ELISA to measure the levels of albumin—an index of permeability injury—and proinflammatory cytokines. The levels of albumin were significantly elevated at 5, 24, and 48 hours, confirming severe alveolar permeability injury immediately after LC. The levels of IL-1 β , IL-6, monocyte chemotactic proteins MCP-1 and MCP-5, macrophage inflammatory protein (MIP)-2, keratinocyte chemoattractant (KC), and tumor necrosis factor (TNF)- α were also measured. The levels of IL-1 β were significantly elevated at 5, 24, and 72 hours after insult. The levels of IL-6 were significantly elevated at 5 hours. The expression of MIP-2 was significantly increased at 5, 24, and 72 hours after LC. There were significant increases in the levels of KC and MCP-1 at 5, 24, and 48 hours after LC. The levels of MCP-5 were significantly increased at 24 and 48 hours. Finally, unlike other primary inflammatory processes involving the lung in which TNF expression is an early response after insult, here there was significantly increased expression of TNF- α at 72 hours after LC (Fig. 3A).

Histological evaluation demonstrated significant injury in *ODD-Luc* mice at all time points after LC. Hemorrhage with alveolar wall necrosis was the predominant lesion. Slides from 3 animals for each group were evaluated by an experienced, blinded pathologist, and examined for the presence of interstitial neutrophil infiltrate, intraalveolar hemorrhage, and pulmonary septal edema. Representative sections were then examined microscopically, and the degree of injury was measured according to the published method (Fig. 3B). This overall profile of inflammation and injury is consistent with that demonstrated in previous mouse and rodent models, substantiating *ODD-Luc* mice as a viable model for LC.^{3,9,13,16,18}

ODD-Luc Mice Lung Lysates Demonstrate Significantly Increased Expression of Hypoxia Responsive Genes

The expression of HIF-1 α in *ODD-Luc* mice was significantly increased at 24 and 48 hours after LC compared with uninjured *ODD-Luc* mice. Moreover, the expression of downstream hypoxia-responsive genes was also significantly elevated. Vascular endothelial growth factor (VEGF)- α , bcl-2 associated x protein (BAX), and IL-6 were increased at 24 and 48 hours after LC. Additionally, the expression of nitric oxide synthase 2 (NOS-2) was elevated at 48 and 72 hours, and the expression of endoplasmic reticulum oxidation 1 (ERO-1) was increased at 24 hours (Fig. 3C). These data indicate that there is significantly increased expression of hypoxia-responsive genes after LC.

Confirmation of Hypoxia in LC

There are many factors that activate HIF-1 α in *ODD-Luc* mice. We therefore performed confirmatory experiments with pimonidazole. We have previously demonstrated that there is global activation of HIF-1 α in *ODD-Luc* mice after LC.¹³ WT mice were injected 1 hour before LC with pimonidazole, a molecule that is reduced in hypoxic environments and subsequently binds thiol-containing proteins. Compared with uninjured controls, WT mice exhibited significantly increased fluorescence at 24 hours after LC, indicating profound hypoxia in both lung and liver (Fig. 4A and B). Next, we determined the distribution of hypoxia among AEC of *ODD-Luc* mice 24 hours after LC. The distribution of bioluminescence was nonuniform. This indicates that hypoxia affects certain AECs preferentially, resulting in a mosaic of hypoxic and normoxic cells (Fig. 4C). Finally, we

measured the expression of HIF-1 α in hepatocytes after LC. The expression of HIF-1 α was significantly higher at 24 hours after LC compared with control (Fig. 4D).

Hypoxic AECs Undergo Apoptosis Without Increased Proliferation

In our previous publication, we determined that HIF-1 α plays a key role in the mediation of the acute inflammatory response after LC.¹³ Here, we sought to determine the fate and importance of hypoxic AECs after LC. Active caspases and DNA fragmentation are characteristic molecular hallmarks of apoptosis. We assessed whether AECs undergo apoptosis using caspase-3 and TUNEL staining. Luciferase dual staining with caspase-3 revealed increased activation of caspase-3 in areas of hypoxia 24 hours after insult compared with uninjured control (Fig. 5A). TUNEL staining also revealed increased apoptotic cells 24 hours after LC compared with uninjured control (Fig. 5C). Additionally, ApoStat was used to detect the presence of active caspases in permeabilized BAL cells. There was a significant elevation in activated intracellular caspases at 24, 48, and 72 hours after LC (Fig. 5D and E). Finally, Ki-67 staining was used to assess the degree of AEC proliferation after LC. There was only minimal Ki-67 staining 24 hours after LC compared with uninjured controls (Fig. 5B). After LC, hypoxic AECs therefore preferentially undergo apoptosis without an associated increase in proliferation.

Hypoxic AECs Significantly Increase Expression of Hypoxia-responsive Genes

Using directly conjugated fluorescent-tagged luciferase antibody, hypoxic cells were visualized and isolated by LCM. Our cell isolation is dependent on luciferase staining and indirect fluorescence LCM, which is a well-established method to identify and isolate specific populations of cells.¹⁹ We have optimized our protocol and collected cells from regions of high fluorescence (hypoxic) and regions without significant fluorescence (normoxic) (Fig. 6A). Using this approach, we separately collected approximately 50 ng of normoxic and hypoxic AECs from regions of low and high fluorescence, respectively, from multiple sections, and repeated the process for 3 different animals. The quality of RNA isolated from these cells was analyzed using Nano Drop spectrophotometer. The expression of HIF-1 α and VEGF in hypoxic and normoxic AECs after LC was analyzed using Q-PCR. The expression of additional genes involved in molecular signaling induced by hypoxia (NOS-2, aryl hydrocarbon receptor nuclear translocator 2 (ARNT-2), and IL-6) was also measured. The expression of VEGF-a is dramatically induced by HIF-1 α in the setting of low oxygen tension in a variety of cell types, and it has been suggested to be not only a key mediator of hypoxia but also a macrophage chemotactic.²⁰ There were significant elevations in the expression of HIF-1 α and VEGF-a in hypoxic compared with normoxic cells. The levels of NOS-2, ARNT-2, and IL-6 were also increased (Fig. 6B). This pattern of hypoxia-related gene expression corresponds to that demonstrated in whole lung lysates, suggesting the main effector cell in hypoxic regulation is at the level of AECs.

Role of Inflammasome Assembly in the Acute Inflammatory Response After LC

Previous work in our laboratory has suggested that IL-1 β is the key intermediate in the regulation of acute inflammation by type II AECs after LC. Subsequent experiments were performed to evaluate the HIF-1 α -induced regulation of IL-1 β and the inflammasome complex. The expression of NLRP-3, a key sensor component of a common inflammasome,

was determined by Western blot in lungs of HIF-1 α (+ / +) and HIF-1 α (– / –) mice at 5, 24, and 48 hours after LC. NLRP-3 was expressed in HIF-1 α (+ / +) mice at all time points, most prominently at 24 hours after LC. HIF-1 α (– / –) mice, however, did not express NLRP-3 at any time point (Fig. 7A).

To evaluate whether hypoxic AECs are preferentially involved in the mediation of the inflammasome complex, the expression of caspase-1 and IL-1 β was evaluated in RNA extracted from hypoxic and normoxic AECs. Caspase-1 is primarily involved in innate immunity as the effector component of the inflammasome complex, and it activates the proinflammatory molecules IL-1 β and IL-18 via proteolytic cleavage.²¹ There were significant elevations in the expression of caspase-1 and IL-1 β RNA in hypoxic cells compared with normoxic cells of *ODD-Luc* mice after LC (Fig. 7B and C). These data suggest that hypoxic activation and subsequent elaboration of HIF-1 α is crucial to inflammasome activation and thereby production of IL-1 β after LC.

DISCUSSION

Lung contusion is a common injury associated with thoracic trauma and a major risk factor for the development of acute respiratory failure such as acute lung injury (ALI)/ARDS. These clinical entities are associated with significant morbidity, as well as considerable increases in intensive care unit (ICU) and hospital length of stay and cost.²² Although more targeted therapeutic strategies continue to be studied, the treatment of LC remains largely supportive. Hypoxia is the most prominent physiologic feature of LC, and we have recently reported that HIF-1 α regulation in type II AECs after LC drives the inflammatory response to injury.¹³ It is particularly difficult not just to measure the precise degree of hypoxia in murine models but also to characterize and study hypoxic cells. Using *ODD-Luc* mice, we demonstrate that the acute inflammatory response in hypoxia reporter mice is similar to that observed in other common species. Additionally, the specific luminescence associated with hypoxic cells in these mice can be used to visualize and study the hypoxic regulation of individual cells. In the present study, we report that after LC, hypoxic AECs specifically undergo apoptosis and trigger downstream pathways related to not only HIF-1 α and hypoxic genes but also the generation of specific intermediates in the inflammasome complex. To date, most models of hypoxia involve the use of a hypoxia chamber. Here, we present an attractive method of studying hypoxia using in vivo animal models of lung injury that reflect actual clinical disease processes that cause hypoxia.

We have previously studied the acute inflammatory changes associated with LC in other mouse models.^{3,9,13,16,18} In the present study, we propose *ODD-Luc* mice as a novel model of LC. Global hypoxia developed after LC and was most pronounced in the liver and spleen. Within the lungs of *ODD-Luc* mice, the pattern of bioluminescence was nonuniform, indicating that activation of HIF is stronger in some AECs compared with others. Pimonidazole staining confirms that hypoxia is essential to this activation. In the present study, we have shown that these hypoxic cells preferentially undergo apoptosis rather than proliferation (Fig. 5). Apoptosis of AECs has been implicated in the pathogenesis of pulmonary fibrosis and lung injury.^{23,24} Additionally, in in vitro studies, it was separately observed that type II AECs exposed to hypoxia undergo apoptosis via HIF-1-mediated up-

regulation of Bnip3L, and that propofol suppresses this pathway.^{8,25} The prominence of caspase-3 staining indicates induction is likely via the extrinsic pathway (Fig. 5A).

Inflammation is most critical to the pathogenesis and outcome of LC. HIF-1 α is now known to be involved in the innate immune response under hypoxic conditions, and this occurs in part by cross-signaling with nuclear factor-kappa B.^{26,27} In the current study, we report that hypoxic AECs after LC preferentially exhibit downstream mediators closely associated with HIF-1 α activation (Fig. 6B). We have previously confirmed in the setting of LC that HIF-1 α inhibition attenuates lung injury and inflammation.¹³ In addition, we reported that IL-1 β is a key player in the initiation of the acute inflammatory response in LC and is directly stimulated by HIF-1 α .¹³ In the current study, we found that regulation of HIF-1 α in type II AECs promotes NLRP-3 inflammasome activation (Fig. 7A). The NLRP-3 inflammasome participates in a variety of infectious and sterile lung diseases, including ALI/ARDS, mostly through antigen-presenting cells.^{11,28} Moreover, the increased expression of caspase-1 and IL-1 β preferentially in hypoxic AECs of *ODD-Luc* mice indicates that hypoxic cells play a central role in the activation of the inflammasome complex and subsequent production of IL-1 β (Fig. 7B and C). Early inhibition of inflammasome activation using small-molecule inhibitors of NLRP-3 may be an attractive target in ARDS, and it has been demonstrated that A438079—an antagonist of the upstream P2X7 receptor—mitigates injury in a mouse model of ALI.^{29,30}

To conclude, we report that *ODD-Luc* hypoxia reporter mice present a unique mechanism to study not only the activation of HIF but also the specific contribution of hypoxic cells to disease processes. Moreover, these data further elucidate the mechanism of lung injury and inflammation induced by contusion. In addition to a focal model of lung injury (LC), we used the hypoxia-reporter mice to document hypoxia using a well-developed model of diffuse bilateral injury (GA). Inhibition of HIF-1 α , in view of its likely role in both apoptosis and inflammasome activation, is thus a promising therapeutic strategy for the treatment of LC.

Acknowledgments

This work was supported by NIH grants R01HL102013 (KR), CA148828 (YS), and DK095201 (YS).

The authors acknowledge receiving the triple transgenic mice HIF-1 α from Dr John J. LaPres, Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan.

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FIGURE 1.

Lung contusion (LC) is characterized by global hypoxia. *ODD-Luc* mice along with uninjured controls were subjected to in vivo imaging system (IVIS) after LC to measure the degree of hypoxia. A, The mice with ODD-linked luciferase showed increased bioluminescence at 24 hours after LC compared with uninjured controls. *ODD-Luc* mice were subjected to LC (a), and explanted abdominal organs were subjected to IVIS to measure the degree of hypoxia. Lung uninjured and LC 24 hours (b), liver uninjured and LC 24 hours (c), and spleen uninjured and LC 24 hours (d). B, Luciferase measurement: luciferase expression in *ODD-Luc* mice was higher in lung and liver after LC (n = 3 per group). (*) $P < 0.05$ WT versus *ODD-Luc* (–/–) mice.

FIGURE 2.

Acid aspiration (GA) is characterized by global hypoxia. *ODD-Luc* mice subjected to gastric acid aspiration (GA) were subjected to in vivo imaging system (IVIS), along with uninjured controls, to assess the degree of hypoxia after insult. There was increased luminescence at all time points after GA compared with control mice. Additionally, explanted organs (lung, liver, kidney, and spleen) were found to have increased luminescence at 24 hours after GA compared with control mice (A–C).

FIGURE 3.

Increased injury and inflammation in ODD-Luc mice after lung contusion. After LC, mice were sacrificed at 5, 24, 48, and 72 hours. BAL albumin and cytokine concentrations were determined by ELISA ($n = 12$ per group). A, ODD-Luc mice showed a significant increase in albumin and cytokine response compared with uninjured ODD-Luc mice after LC: the levels of albumin, IL-1 β , IL-6, MCP-1 (CCL2), MCP-5 (CCL12), MIP-2, KC, and TNF- α in the BAL were significantly higher after LC compared with uninjured mice ($n = 12$). B, Statistical analysis was performed with 2-tailed unpaired t test with Welch correction. (*) $P < 0.05$ ODD-Luc mice versus uninjured ODD-Luc mice. Histological evaluation of ODD-Luc mice after LC: in WT mice, there were large areas of hemorrhage at 5 hours with increasing necrosis and inflammation at 24, 48, and 72 hours after LC compared with corresponding control mice ($n = 3$ per group). C, Whole lung lysates demonstrate significantly increased expression of hypoxia-related genes: the expression of HIF-1 α , VEGF-a, BAX, NOS, IL-6, and ERO-1 was significantly higher in ODD-Luc mice after LC compared with uninjured ODD-Luc mice ($n = 3$ per group). (*) $P < 0.05$ ODD 0 hour versus ODD-Luc (-/-) mice.

FIGURE 4.

Wild-type (WT) mice undergo predominant hypoxia expression after LC. WT mice of C57BL/6 background were subjected to LC (n = 3 per group). A and B, Lung and liver samples were harvested at 24 hours and subsequently subjected to immunofluorescent staining for hypoxia (green) and nuclear staining with DAPI (4',6-diamidino-2-phenylindole) (blue). C, *ODD-Luc* mice show elevated expression of luciferase after LC. Frozen sections from *ODD-Luc* mice were harvested 24 hours after LC and subjected to immunofluorescent staining with luciferase (green) and DAPI (blue). D, Lung and liver samples were harvested at 24 hours after LC and subjected to immunofluorescent staining for HIF-1α.

FIGURE 5.

ODD-Luc mice undergo apoptosis without significantly increased proliferation after LC. A, Luciferase dual-staining with caspase-3. Frozen lung sections from *ODD-Luc* mice were harvested after LC and subjected to immunofluorescent staining with luciferase (green), caspase-3 (red), and DAPI (blue) ($n = 3$). C, Ki67 expression. Immunofluorescence analyses of Ki67 were performed. For staining, cells were fixed and subsequently incubated with anti-luciferase (green), anti-Ki67 Ab (red), and DAPI (blue) ($n = 3$). B, TUNEL staining in *ODD-Luc* mice after LC. After LC, frozen sections were collected, fixed, and stained with TUNEL Apoptosis Detection Kit and apoptotic cells (green) and DAPI (blue) ($n = 3$). D and E, ApoStat. BAL fluid from *ODD-Luc* mice was collected at different time points after LC. Increased active caspases were found at all the time points compared with uninjured *ODD-Luc* mice. Statistical analysis was performed with 2-tailed unpaired t test with Welch correction. (*) $P < 0.05$ *ODD-Luc* mice versus uninjured *ODD-Luc* mice.

FIGURE 6.

Quantitative TaqMan PCR analysis after laser capture microdissection (LCM). LCM is a method to procure subpopulations of tissue cells under direct microscopic visualization. Luciferase staining of *ODD-Luc* frozen section reveals areas of viable hypoxic and normoxic cells (A-a). *ODD-Luc* lung sections before and after LCM with labeled normoxic (blue circle) and hypoxic (pink circle) regions (A-b). Hypoxic and normoxic regions excised from lung sections (A-c). The expression of various classic hypoxic markers was measured in *ODD-Luc* mice after LC. Real-time PCR analysis demonstrated significantly increased levels of HIF-1 α , VEGF-a, NOS-2, ARNT-2, and IL-6 in hypoxic AECs after LC (n = 5) (B). (*) $P < 0.05$ hypoxic versus normoxic cells.

FIGURE 7.

Inflammasome signaling activation after LC. To determine inflammasome downstream signaling activation in HIF-1 α (+ / +) and HIF-1 α (- / -) mice after LC, nuclear extracts were analyzed with Western blot using various antibodies (n = 3 per group). HIF-1 α (+ / +) mice showed significant induction of NLRP-3 (A). Real-time PCR analysis demonstrated a significant increase in the levels caspase-1 and IL-1 β in hypoxic cells after LC (n = 3 per group) (B and C). (*) $P < 0.05$ hypoxic versus normoxic cells.