Small interfering RNA targeting heme oxygenase-1 enhances ischemia-reperfusion-induced lung apoptosis

Xuchen Zhang¹, Peiying Shan¹, Dianhua Jiang¹, Paul W. Noble¹, Nader G. Abraham², Attallah Kappas³, and Patty J. Lee¹#

¹Section of Pulmonary and Critical Care Medicine, Yale University School of Medicine, New Haven, Connecticut 06520, USA, ²Department of Pharmacology, New York Medical College, Valhalla, New York 10595, USA, and ³The Rockefeller University, New York, New York 10021, USA.

Running Title: Small-interfering RNA targeting heme oxygenase-1

# Corresponding Author:
Patty J. Lee M.D.
Section of Pulmonary & Critical Care Medicine
Yale University School of Medicine
P.O. Box 208057
New Haven, CT 06520-8057, USA

Telephone: 1-203-785-5877  Fax: 1-203-785-3826

E-mail: patty.lee@yale.edu
Abstract

Heme oxygenase-1 (HO-1) is emerging as an important cytoprotective enzyme system in a variety of injury models. In order to optimize future therapeutic applications of HO-1, it is necessary to delineate precise functions and mechanisms as well as modes of externally regulating HO-1 expression. Investigations have been limited by difficulties with the generation of HO-1 null mice and the lack of specific HO-1 inhibitors. Lung ischemia-reperfusion (I-R) injury is the inciting event in acute lung failure following transplantation, surgery, and shock. In order to study the function of HO-1 in I-R-induced lung injury, we designed small interfering RNA (siRNA) sequences that effectively suppress HO-1 expression both in vitro and in vivo in an organ-specific manner. In this study we show that there is enhanced apoptosis, via increased Fas expression and caspase 3 activity, in the presence of HO-1 siRNA in endothelial cells and mouse lung during I-R injury, whereas HO-1 overexpression attenuates apoptosis. To the best of our knowledge, we are the first to demonstrate that lung-specific siRNA delivery can be achieved by intranasal administration without the need for viral vectors or transfection agents in vivo, thereby obviating potential concerns of toxicity if siRNA technology is to have clinical application in the future.

Key Words: small interfering RNA, heme oxygenase-1, apoptosis, caspases, Fas
**Introduction**

HO-1 is one of three isoforms of HO, the rate-limiting enzyme in the degradation of heme to biliverdin and eventually, to bilirubin. HO-1 expression is induced in multiple cell types and organs in response to injury. This induction is postulated to have protective properties, however, the mechanisms remain elusive (1-4). HO-2 is primarily constitutive and has been found to be important in the central nervous system (5). The function of HO-3 is yet unknown. Synthetic heme analogues such as protoporphyrins that competitively inhibit the activity of all HO isoforms are commonly used to study HO-1 function but are limited by the lack of specificity and can have the paradoxical effect of upregulating HO-1 protein expression (6,7). The HO inhibitor tin mesoporphyrin is approved by the Federal Drug Administration for the treatment of hyperbilirubinemia but, again lacks specificity (8). The recent emergence of siRNA technology to silence mammalian genes allows for highly specific analysis of gene function and has potential clinical application. Among the limited number of reports on siRNA administration in vivo, all use systemic delivery, transfection chemicals or viral vectors and none have been organ-specific, to the best of our knowledge (9-13). We demonstrate that intranasal siRNA delivery, without a vector or transfection agent, has lung-specificity and that HO-1 potently regulates lung apoptosis. Lung-specific siRNA will not only be a useful tool to study gene function but may have therapeutic applications for a wide range of lung diseases. Furthermore, HO siRNA may become the basis of modulating severe hyperbilirubinemia of newborns and the severe jaundice of Crigler Najjar Type I patients, where there is excessive bilirubin formation and for whom specific therapy does not currently exist.
Experimental Procedures

PAEC anoxia-reoxygenation (A-R) and mouse lung I-R models. Primary rat pulmonary artery endothelial cells (PAEC) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, GibcoBRL, Grand Island, NY, USA) with 10% FBS (Hyclone, Logan, UT, USA), and 0.1% gentamicin (GibcoBRL). Dr. Troy Stevens, University of Alabama, generously provided the PAEC. All data using PAEC were collected before passage 20. PAEC were exposed to anoxia (95% N₂/5% CO₂) in a sealed modular chamber (Billup-Rothberg, Del Mar, CA, USA) with continuous monitoring and automated adjustments to maintain <0.5% O₂ (BioSpherix, Redfield, NY, USA) for 24 hours, as previously described (14). For anoxia-reoxygenation (A-R), after 24 hours of anoxia, the media was changed and cells were exposed to normoxia at 37°C in a humidified atmosphere of 5% CO₂ for 1 hour. For mouse lung I-R, after an intraperitoneal injection of urethane (180 mg/kg body weight), the mice were intubated via tracheostomy, and ventilated with Harvard ventilator (respiratory rate = 120/minutes, tidal volume = 0.5 cm). A left hilar clamp was placed for 30 minutes of unilateral ischemia to the left lung and the clamp was then released for 2 hours of reperfusion as previously described (14). All mouse experiments use male C57Bl/6J mice, 6-8 weeks of age weighing 20-25 gm, purchased from Jackson Laboratory (Bar Harbor, ME, USA). The Animal Care and Use Committee at Yale University approved this protocol in accordance with their guidelines.

siRNA design and preparation. The design of siRNAs was based on the characterization of siRNA by Elbashir et al. (15,16). siRNAs were synthesized in 2’-Deprotected, Duplexed, Desalted, and Purified form by Dharmacon Research, Inc. (Lafayette, CO, USA). The sense and anti-sense strands of rat and mouse heme oxygenase-1 (HO-1) siRNA were: sequence 1: sense: 5’-AAGGACAUGGCUCCUGGUAUdTdT-3’, antisense: 5’-AUACCAGAAGCCAUGGCUCCUdTdT-3’; sequence 2: sense: 5’-AAUGAACACUGGAGAUGACdTdT-3’, antisense: 5’-GUCAUCUCAGAGUGUUAUUdTdT-3’; sequence 3: sense: 5’-AAGACCAGAGUCCUCACAGAddTdT-3’, antisense: 5’-
UCUGUGAGGGACUCUG GUCU UdTdT-3'; sequence 4: sense: 5’-
AAGCCACACGCAUCUAUGUAAdTdT-3’, antisense: 5’-UUACAUAGUGCUGUGUG
GCU UdTdT-3’; Sequence 5: sense: 5’-AAGCCAGAAUGCAGUUCAdTdT-3’, antisense: 5’-
UGAACUGCAUUCUG CCGCU UdTdT-3’. The sense and anti-sense strands of human heme
oxygenase-1 (HO-1) siRNA were: sequence 1: sense: 5’-GGAGAUUGAGCGCAAAAGdTdT-
3’, anti-sense: 5’-CUUGUGCGCUAAAU CUCdCdT-3’; sequence 2: sense: 5’-
UGAUAGAAGAGGGCAAGACdTdT-3’, anti-sense: 5’-GUCUUGGCUCUUCAUCAAdTdT-3’;
sequence 3: sense: 5’-CUGCUUCCUGCUAACAAdTdT-3’, anti-sense: 5’-
AUGUUGAGCAGGAACG CAGdTdT-3’. 5’-Biotin-labelled rodent HO-1 siRNA sequence 4 and
nonspecific siRNA scrambled duplex (sense: 5’-GCGCGCUUUGUGGAAUCGdTdT-3’, anti-
sense: 5’-CGAAUCCAAAGCGCGCdTdT-3’) were also synthesized by Dharmacon
Research, Inc. Nonspecific control 5’-fluorescein labeled siRNA (sense: 5’-
UUCUCCAAGGUGUCACGdTdT-3’, anti-sense: 5’-ACGUGACAGGUA CGGA GAAdTdT-3’)
was synthesized by Qiagen (Germantown, MD, USA).

Transfection of siRNA duplexes in vitro and in vivo. PAEC were seeded into 6- or 12-
well plates one day prior to transfection using DMEM tissue culture medium supplemented with 10%
FBS, without antibiotics. At the time of transfection with siRNA, the cells were 50-60% confluent.
Oligofectamine Reagent (Invitrogen, Carsbad, CA, USA) was used as the transfection agent
and cells were then incubated for 6 hours. Next, 30% FBS DMEM medium was added to reach
a final concentration of 10% FBS in the wells. Cells were exposed to A-R 24 hours after
transfection. For in vivo studies, each mouse was anesthetized with methoxyflurane and then
given intranasal HO-1 siRNA (2 mg/kg body weight) or equivalent doses of nonspecific control
siRNA duplex or recombinant adenovirus containing rat HO-1 cDNA (Ad-HO-1, a generous gift
from Dr. Leo Otterbein, University of Pittsburgh) or the recombinant adenovirus containing the
β-galactosidase gene (Ad-X-LacZ, purchased from BD Biosciences Clontech, Palo Alto, CA,
USA) in a volume of 50 µl.
Generation of stable PAEC cell line overexpressing human HO-1. The human HO-1-expressing replication-deficient retrovirus vector LSN-HHO-1 has been previously described (17,18). Exponentially growing PA317 packaging cells were used for transfection and preparation of viral particles. Individual G418-resistant clones were selected and initial viral titer assays were measured by infecting NIH-3T3 cells as previously described (18). PA317/LSN-HHO-1 and the empty viral control cells, PA317/LXSN, were grown until subconfluence. The supernatants were harvested and used to infect PAEC. After selection with G418 a stably transfected cell line of PAEC overexpressing human HO-1, designated LSN/HO-1, and a retroviral vector control cell line, LXSN, were obtained.

Western blot analysis. As previously described (14) protein was extracted from cell or lung tissue lysates, electrotransferred, and then immunoblotted with monoclonal HO-1 antibody (Stressgen Biotechnologies, Victoria, BC, Canada). Detection was performed with Phototope-HRP western detection system (Cell Signaling Technology, Beverly, MA, USA). Equivalent sample loading was confirmed by stripping membranes with Blot Restore Membrane rejuvenation solution (Chemicon International, Inc., Temecula, CA, USA) and re-probed with anti-β-tubulin antibody.

Apoptosis Assays. We used fluorescence-activated cell sorter (FACS) to detect Annexin V-FITC labeling (BD Pharmingen, San Diego, CA, USA) according to the manufacturer’s instruction. Briefly, after PAEC were washed with cold PBS and resuspended with binding buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2), a solution containing 1×10^5 cells was transferred to a 5 ml tube, and 5 μl of Annexin V and 5 μl of Propidium iodide (PI) were added. Binding buffer was then added to each tube and analyzed by FACS (Becton Dickinson, San Jose, CA, USA). The Annexin V-FITC signal was detected by FL1 (FITC detector) at 518 nm, and the PI signal was detected by FL2 (phycoerythrin fluorescence detector) at 620 nm. Mouse lung sections were subjected to terminal deoxynucleo-
tidyltransferase dUTP nick end-labeling (TUNEL) assay using the *in situ* cell death detection kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) as previously described (19).

*Fas expression and caspase 3 activity.* For Fas expression PAEC were washed twice in cold PBS, pelleted, suspended in PBS containing Fas (1:100 dilution) or control rat IgG (1:100 dilution) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and incubated on ice for 45 minutes. The cells were resuspended in PBS containing anti-rat-FITC (1:50 dilution) antibody (Santa Cruz Biotechnology) and fixed in 1% paraformaldehyde prior to FACS analysis. Caspase 3 activity was measured with colorimetric assays using CaspACE Assay System (Promega, Madison, WI, USA). PAEC lysates were centrifuged and the supernatants were incubated with colorimetric substrate, Ac-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA). The release of p-nitroanilide (pNA) from Ac-DEVD-pNA was measured at 405 nm using a spectrophotometer.

*Statistics.* Data are expressed as mean ± S.E. and analyzed by student’s *t*-test. Significant difference was accepted at *P*<0.05.
Results

**HO-1 siRNA inhibits A-R induced HO-1 protein expression in PAEC.** We have previously shown that mouse lungs subjected to I-R injury or endothelial cells exposed to A-R induce HO-1 expression (14). However, the function of HO-1 during lung I-R injury is unknown. In order to delineate the role of HO-1 in lung I-R injury, we sought to knock down HO-1 induction *in vitro* and *in vivo* using HO-1 siRNA. We designed five siRNA sequences (1 to 5) directed against rodent HO-1, according to the methods of Elbashir et al. (20), and transfected them into PAEC. We determined that sequence 4 was the most effective in inhibiting A-R-induced HO-1 protein expression (Fig. 1a). The silencing effect of sequence 4 on HO-1 protein induction during A-R was dose-dependent, whereas incremental doses of non-specific siRNA had no effect on HO-1 expression (Fig. 1b). Using 5'-biotin-labeled HO-1 siRNA, we demonstrated efficient entry of the siRNA into PAEC as denoted by the diffuse brown staining in the cytoplasm (Figure 1c). We also quantitated transfection efficiency to be greater than 88%, using 5'-fluorescein labeled siRNA detection by FACS (data not shown). All subsequent *in vitro* and *in vivo* studies utilized sequence 4 HO-1 siRNA.

**HO-1 siRNA inhibits I-R-induced HO-1 protein expression in mouse lung.** We tested the efficacy of the HO-1 siRNA in mouse lung by intranasal administration. A time course of HO-1 siRNA delivery prior to subjecting the mouse to lung I-R injury shows that HO-1 siRNA has maximal effect in attenuating HO-1 protein induction if given 16 hours prior to I-R injury (Figure 2a, lane 5) and persists for at least 72 hours (data not shown). Therefore, intranasal HO-1 siRNA was given for 16 hours prior to I-R lung injury in the subsequent mouse experiments. Lung I-R injury causes HO-1 protein induction in other organs such as kidney, liver and heart, but intranasal siRNA administration is lung-specific (Figure 2b, lane 3). As expected, intranasal administration of nonspecific siRNA, at the same dose, had no effect on I-R-induced HO-1 expression in lung or other organs (Figure 2b, lane 4). We confirmed lung-delivery of the siRNA by intranasally administering a 5'-biotin-labeled HO-1 siRNA that confers a brown stain to cells
that have incorporated the siRNA. We detected brown-stained cells diffusely in the airway and lung parenchyma as early as 4 hours and persisting to 16 hours (Figure 2c).

**HO-1 overexpression in PAEC and mouse lung.** In order to contrast the effects of HO-1 siRNA with HO-1 overexpression, we used human HO-1 gene in a replication-defective retroviral vector, which has been previously described (18), to stably transfect PAEC. We achieved significantly increased HO-1 protein expression in the HO-1 overexpressors (LSN/HO-1) compared to empty vector transfection (LXSN) in room air (Figure 3a). We used intranasal administration of rat HO-1 adenovirus (Ad-HO-1) to achieve HO-1 overexpression in mouse lungs. We determined HO-1 protein levels at 48 hours, 72 hours, 5 days, and 1 week after administration of Ad-HO-1 and observed increased HO-1 protein after 48-72 hours (Figure 3b, Upper Panel, lanes 2 and 3). As expected, the empty vector (Ad-X-LacZ) had no effect on HO-1 induction in mouse lung (Figure 3b, Lower Panel, lane 3). In order to effectively silence the human HO-1 gene in the stably transfected PAEC, we designed three human HO-1 siRNA sequences (1 to 3). Transfection experiments revealed that all three siRNA sequences, especially at higher doses, inhibited HO-1 expression in HO-1 overexpressing PAEC if given for 24-72 hours (Figure 3c). At 72 hours of siRNA transfection, lower doses (300 nM) of siRNA appeared to be less effective in suppressing HO-1 protein, likely due to degradation of the siRNA. Sequence 1 at 600 nM for 24 hours was used in subsequent experiments with human HO-1 overexpressing PAEC (LSN/HO-1).

**HO-1 modulates apoptosis in PAEC and mouse lung during A-R and I-R injury, respectively.** Apoptosis is a pivotal mechanism of I-R-induced organ injury (21-23). We demonstrated the biological effect of HO-1 on apoptosis in PAEC during A-R and in mouse lung during I-R. When we knocked down HO-1 expression in PAEC with siRNA directed against rodent HO-1, there was a dramatic increase in A-R-induced apoptosis in PAEC (quantitated by FACS analysis) to 33.8±2.2% during anoxia alone and 41.3±1.4% during A-R compared to wildtype controls and cells transfected with non-specific siRNA (Figure 4a, lanes 1-3). Given that the absence of HO-
1 induction during A-R was deleterious, we expected that HO-1 overexpression would attenuate apoptosis. HO-1 overexpressing cells (LSN/HO-1) subjected to A-R exhibited significantly less apoptosis (8.6±0.2%) during A-R compared to wildtype controls and cells that express empty-vector (LXSN) (Figure 4a, lanes 1, 4, and 5). This effect on apoptosis appeared to be specific to HO-1 because we were able to reverse the anti-apoptotic effect of HO-1 overexpression by introducing HO-1 siRNA directed against the human HO-1 overexpressing vector (Figure 4a, lane 6). However, given that the HO-1 siRNA was directed against the exogenously transfected human HO-1 gene and considering our previous demonstration that there was endogenous induction of rat HO-1 in PAEC (a rat cell line) during A-R injury (14), we proceeded to simultaneously inhibit both rodent and human HO-1. Notably, the presence of both rodent and human siRNA led to more apoptosis than human siRNA alone (41.4±2.4% versus 26.8±0.7%) (Figure 4a, lanes 6 and 7, respectively). This was likely due to the fact that even in the presence of human siRNA, which only silenced the transfected human HO-1 gene expression, endogenous HO-1 induction in the PAEC overexpressors continued to have an anti-apoptotic effect. Only when both the exogenous human HO-1 and the endogenous HO-1 induction were silenced did apoptosis reach the levels seen in wildtype PAEC treated with rodent HO-1 siRNA (Figure 4a, lane 3). Nonspecific siRNA transfection at equivalent doses had no effect on A-R-induced apoptosis. These findings demonstrated the species-specificity of the siRNA sequences and highlighted the anti-apoptotic effect of HO-1. We proceeded to correlate our in vitro findings with in vivo studies by subjecting mice to lung I-R injury and assessing TUNEL staining. HO-1 siRNA significantly increased the number of TUNEL-positive cells compared to naïve mice, mice subjected to I-R alone, and mice with nonspecific siRNA subjected to I-R (Figure 4b, panels A-D). On the other hand, mice that overexpressed HO-1 in the lung (Ad-HO-1, panel F) exhibited significantly less TUNEL staining during lung I-R compared to mice subjected to I-R alone and mice given empty vector (Ad-X-LacZ ) (Figure 4b, panels B and E,
respectively). The quantitation of TUNEL positive cells paralleled the PAEC results in that HO-1 siRNA in vivo caused significantly increased lung apoptosis, whereas HO-1 overexpression with intranasal administration of HO-1 adenoviral vector decreased apoptosis during lung I-R (Figure 4c). Taken together, our data indicate that in both endothelial cells and mouse lung, HO-1 expression has profound anti-apoptotic properties during A-R and I-R injury, respectively.

**HO-1 modulates apoptosis via Fas and caspase 3-dependent mechanisms in PAEC during A-R injury.** We have previously demonstrated that Fas/FasL expression and caspase 3 activity are increased in PAEC during A-R (24). In order to delineate potential mechanisms of HO-1-modulated apoptosis, we investigated the effect of HO-1 siRNA and overexpression on Fas and caspase 3 expression in PAEC during A-R. Figure 5a is a representative FACS analysis of Fas expression in PAEC during A-R which demonstrated that HO-1 siRNA increased A-R-induced Fas expression whereas stable HO-1 overexpression decreased Fas expression. Antibody to rat IgG was used as a negative control. Figure 5b is a graphical representation of three independent FACS analyses of Fas expression. The Y-axis depicts the percentage of cells, compared to the total, that express Fas during the various conditions. In the presence of HO-1 siRNA (Figure 5b, lane 3), there was significantly increased Fas expression (50.2±2.3%) compared to the wildtype control cells (31.6±0.9%) and cells transfected with nonspecific siRNA (30.4±1.6%) during A-R. However, in the presence of stable HO-1 overexpression using human HO-1 gene (LSN/HO-1), there was a dramatic attenuation of A-R-induced Fas expression to 16.9±1.3% compared to cells stably transfected with empty vector (LXSN) (Figure 5b, lanes 4 and 5). We were able to reverse the effects of HO-1 overexpression on Fas expression by transfecting the HO-1 overexpressors with siRNA directed against the human HO-1 vector (Figure 5b, lane 6). In parallel with our apoptosis studies in PAEC (Figure 4a), the silencing of both the human HO-1 overexpression vector and the endogenous rodent HO-1 induction in PAEC resulted in even greater Fas expression in PAEC during A-R (Figure 5b, lane 7). In
addition to Fas, HO-1 also modulated caspase 3 activity during A-R. HO-1 overexpression significantly attenuated A-R-induced caspase 3 activity in PAEC (1.6±0.1%) (Figure 5c, lane 5). On the other hand, HO-1 siRNA, especially if directed against both the exogenous human HO-1 vector and endogenous rodent HO-1, dramatically increased caspase 3 activity in PAEC during A-R (4.6±0.2%) (Figure 5c, lane 7).
Discussion

Our studies are the first to utilize the highly specific technology of siRNA to directly demonstrate that HO-1 has a dramatic effect on apoptosis during lung I-R injury, via Fas and caspase 3-dependent pathways. HO-1 overexpression by gene transfer successfully attenuated I-R-induced apoptosis and may potentially have therapeutic value in I-R-induced lung injury, for which specific therapies do not currently exist. Lung I-R injury is critical to the pathogenesis of acute lung injury during lung transplantation/surgery, pulmonary embolism, and re-expansion pulmonary edema. The inhibition of apoptosis during I-R injury in other organ systems has been shown to be cytoprotective and promote organ survival (25,26). Therefore, identifying genes that modulate I-R-induced apoptosis would have potential therapeutic relevance to a variety of organ systems. In addition, we demonstrate that lung-specific siRNA delivery, without the presence of viral vectors or transfection agents, is biologically effective and may have significant implications for future therapeutic interventions.

Our data show that the transfer of exogenous human HO-1 cDNA to endothelial cells or adenoviral-mediated HO-1 overexpression in mouse lung dramatically attenuates A-R/I-R-induced apoptosis. Conversely, specific knockdown of HO-1 expression using siRNA \textit{in vitro} and \textit{in vivo} significantly increased A-R/I-R-induced apoptosis. Furthermore, we use HO-1 overexpression vectors and HO-1 siRNA to demonstrate that HO-1 specifically modulates endothelial cell apoptosis by attenuating A-R-induced Fas expression and caspase 3 activity. Thus far, most investigations into the consequences of HO-1 deficiency have utilized synthetic heme analogues such as the protoporphyrins, which are not specific for HO-1 and have the paradoxical effect of inducing HO-1 expression (6,7). Reports using HO-1-deficient mice have been limited, likely due to breeding difficulties, and dominant-negative constructs have not been effective. The use of HO-1 siRNA will greatly facilitate the precise identification of HO-1 gene function.
We show that HO-1 siRNA has biologic function both *in vitro* as well as *in vivo* and can be effective in an organ-specific manner. RNA interference (RNAi) mediated by siRNA is a powerful technology that allows the silencing of genes with great specificity and potency. Although used extensively in plants, nematodes, and Drosophila to determine gene function, until recently RNAi was not applicable in mammalian cells. The discovery that transfection of short 21-23 nucleotide siRNA into mammalian cells specifically interferes with gene expression and does not induce nonspecific responses opened the technique to the study of mammalian gene function and potential therapeutic applications (20). There are increasing reports on the use of siRNA *in vitro* (27-30). However the *in vivo* use of siRNA has been limited. Researchers have found that systemic delivery of caspase 8 siRNA or Fas siRNA into mice protects against liver injury (13,31). Similarly, McCaffrey et al. showed that the administration of hepatitis B virus (HBV) siRNA effectively inhibited HBV replication in cultured cells and mouse liver, indicating that siRNA could be useful in the treatment of viral liver diseases (32). Subretinal siRNA can also be achieved using a chemical transfection agent (11), and transgenic approaches as well as viral-constructs have been used to deliver siRNA *in vivo* (9,33). The reports thus far of siRNA *in vivo* utilize systemic delivery of the siRNA and/or require the use of a transfection chemical or viral vector, which potentially raises concerns for toxicity if used clinically. Furthermore, although systemically-delivered siRNA can be detected in multiple organs, including lung, it is clear that the biologic activity of systemically-administered siRNA is not equally effective in all organs. For example, systemic injection of caspase 8 siRNA, although detected in most organs, inhibited Fas-induced liver apoptosis but not lung or kidney apoptosis (13).

In our present study, intranasal administration of HO-1 siRNA, in the absence of transfection agents, had significant lung-specific effects in modulating apoptosis. We observed the presence of HO-1 siRNA diffusely in the lung airway and alveoli. In the near future the use of tissue-specific siRNA may become feasible (with pol II rather than pol III promoters), opening
up the possibility of targeting specific lung cell types. The ability to apply siRNA in an organ or cell-specific manner will be of paramount interest not only for lung diseases but also for a broad range of clinical processes.
Acknowledgements: PJL is supported by National Institutes of Health (NIH) Grant HL004034 and American Heart Association Grant 0355863T; NGA is supported by NIH Grants HL55601, HL31069, and HL34300.
References

1. Otterbein, L. E., and Choi, A. M. (2000) Am. J. Physiol. Lung Cell Mol. Physiol. 279, L1029-1037

2. Otterbein, L. E., Kolls, J. K., Mantell, L. L., Cook, J. L., Alam, J., and Choi, A. M. K. (1999) J. Clin. Invest. 103, 1047-1054

3. Abraham, N. G., Lavrovsky, Y., Schwartzman, M. L., Stoltz, R. A., Levere, R. D., Gerritsen, M. E., Shibahara, S., and Kappas, A. (1995) Proc Natl Acad Sci U S A 92, 6798-6802

4. Lee, P. J., Otterbein, L. E., Sethi, J., Sasidhar, M., and Choi, A. M. K. (2002) in Disease markers in exhaled breath (Marczin, N., Kharitonov, S., Yacoub, M., and Barnes, P., eds) Vol. 170, pp. 117-127, Marcel Dekker, New York

5. Dore, S., Takahashi, M., Ferris, C. D., Hester, L. D., Guastella, D., and Snyder, S. H. (1999) Proc Natl Acad Sci USA 96, 2445-2450

6. Jozkowicz, A., and Dulak, J. (2003) Acta Biochim Pol 50, 69-79

7. Sardana, M. K., and Kappas, A. (1987) Proc Natl Acad Sci U S A 84, 2464-2468

8. Kappas, A. (2002) in Studies from the laboratory bench to newborn nursery in heme oxygenase in biology and medicine (NG Abraham, J. A., and KA Nath, ed), pp. 3-17, Kluwer Academic/Plenum publishers

9. Hasuwa, H., Kaseda, K., Einarsdottir, T., and Okabe, M. (2002) FEBS Lett 532, 227-230

10. McCaffrey, A. P., Meuse, L., Pham, T. T., Conklin, D. S., Hannon, G. J., and Kay, M. A. (2002) Nature 418, 38-39

11. Reich, S. J., Fosnot, J., Kuroki, A., Tang, W., Yang, X., Maguire, A. M., Bennett, J., and Tolentino, M. J. (2003) Mol Vis 9, 210-216

12. Sorensen, D. R., Leirdal, M., and Sioud, M. (2003) J Mol Biol 327, 761-766
13. Zender, L., Hutker, S., Liedtke, C., Tillmann, H. L., Zender, S., Mundt, B., Waltemathe, M., Gosling, T., Flemming, P., Malek, N. P., Trautwein, C., Manns, M. P., Kuhnel, F., and Kubicka, S. (2003) Proc Natl Acad Sci U S A 100, 7797-7802
14. Zhang, X., Bedard, E. L., Potter, R., Zhong, R., Alam, J., Choi, A. M., and Lee, P. J. (2002) Am J Physiol Lung Cell Mol Physiol 283, L815-829
15. Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001) Genes Dev 15, 188-200
16. Elbashir, S. M., Harborth, J., Weber, K., and Tuschl, T. (2002) Methods 26, 199-213
17. Quan, S., Yang, L., Abraham, N. G., and Kappas, A. (2001) Proc Natl Acad Sci U S A 98, 12203-12208
18. Yang, L., Quan, S., and Abraham, N. G. (1999) Am J Physiol 277, L127-133
19. Zhang, X., Shan, P., Otterbein, L. E., Alam, J., Flavell, R. A., Davis, R. J., Choi, A. M., and Lee, P. J. (2003) J Biol Chem 278, 1248-1258
20. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Nature 411, 494-498
21. Stammberger, U., Gaspert, A., Hillinger, S., Vogt, P., Odermatt, B., Weder, W., and Schmid, R. A. (2000) Ann Thorac Surg 69, 1532-1536
22. Cao, G., Pei, W., Ge, H., Liang, Q., Luo, Y., Sharp, F. R., Lu, A., Ran, R., Graham, S. H., and Chen, J. (2002) J Neurosci 22, 5423-5431
23. Calvillo, L., Latini, R., Kajstura, J., Leri, A., Anversa, P., Ghezzi, P., Salio, M., Cerami, A., and Brines, M. (2003) Proc Natl Acad Sci U S A 100, 4802-4806
24. Zhang, X., Shan, P., Alam, J., Davis, R. J., Flavell, R. A., and Lee, P. J. (2003) J Biol Chem 278, 22061-22070
25. Chanalaris, A., Sun, Y., Latchman, D. S., and Stephanou, A. (2003) J Mol Cell Cardiol 35, 257-264
26. Yaoita, H., Ogawa, K., Maehara, K., and Maruyama, Y. (1998) Circulation 97, 276-281
27. Shen, C., Buck, A. K., Liu, X., Winkler, M., and Reske, S. N. (2003) *FEBS Lett* **539**, 111-114

28. Barton, G. M., and Medzhitov, R. (2002) *Proc Natl Acad Sci U S A* **99**, 14943-14945

29. Devroe, E., and Silver, P. A. (2002) *BMC Biotechnol* **2**, 15

30. Abbas-Terki, T., Blanco-Bose, W., Deglon, N., Pralong, W., and Aebischer, P. (2002) *Hum Gene Ther* **13**, 2197-2201

31. Song, E., Lee, S. K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P., and Lieberman, J. (2003) *Nat Med* **9**, 347-351

32. McCaffrey, A. P., Nakai, H., Pandey, K., Huang, Z., Salazar, F. H., Xu, H., Wieland, S. F., Marion, P. L., and Kay, M. A. (2003) *Nat Biotechnol* **21**, 639-644

33. Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., Scott, M. L., and Van Parijs, L. (2003) *Nat Genet* **33**, 401-406
Figure legends

**Figure 1.** HO-1 siRNA inhibited HO-1 protein expression in PAEC during A-R. a, PAEC were transfected with different HO-1 siRNA sequences (1 to 5) prior to A-R injury and immunoblotted against HO-1 or β-tubulin (loading control). RA, room air control; A-R, 24 hours anoxia followed by 1 hour reoxygenation; lanes 1 to 5 represent HO-1 siRNA sequences 1 to 5. b **Upper Panel,** PAEC were transfected with varying doses of HO-1 siRNA (sequence 4) prior to A-R injury and immunoblotted against HO-1 or β-tubulin (loading control). b **Lower Panel,** PAEC were transfected with varying doses of nonspecific siRNA prior to A-R injury and immunoblotted against HO-1 or β-tubulin (loading control). c, PAEC were transfected with 5′-biotin-labeled HO-1 siRNA (siRNA-Biotin) for 24 hours and then incubated with peroxidase-labeled streptavidin followed by DAB as substrate. Brown-staining indicates the presence of HO-1 siRNA.

**Figure 2.** HO-1 siRNA inhibited HO-1 protein expression in mouse lung during I-R. a, Wildtype mice were administered intranasal rodent HO-1 siRNA (2mg/kg body weight) 4, 8, and 16 hours before I-R, and the lung lysates were immunoblotted against HO-1 or β-tubulin (loading control). I-R, 30 minutes of left lung ischemia followed by 2 hours of reperfusion. b, Tissue lysates from the indicated organs were extracted after intranasal administration of rodent HO-1 siRNA or nonspecific siRNA for 16 hours prior to lung I-R injury. Lysates were then immunoblotted against HO-1 or β-tubulin (loading control). c, Mice were administered intranasal 5′-biotin labeled rodent HO-1 siRNA for 4 hours (panel B) or 16 hours (panel C). Lung sections were then incubated with peroxidase-labeled streptavidin and DAB substrate to assess for the presence of biotin (brown staining). Brown-staining indicates the presence of HO-1 siRNA. Double arrows, airway; single arrow, alveolae.

**Figure 3.** HO-1 overexpression in PAEC and mouse lung. a, Cell lysates from PAEC stably transfected with human HO-1 gene in a replication-deficient retroviral vector (LSN/HO-1) or
empty vector (LXSN) were immunoblotted against HO-1 or β-tubulin (loading control). b Upper Panel, Mice were administered intranasal rat HO-1 gene in an adenoviral vector (Ad-HO-1, 5x10⁸ pfu) and lung lysates were obtained after 48 hours, 72 hours, 5 days, and 1 week. The lung lysates were then immunoblotted against HO-1 or β-tubulin (loading control). b Lower Panel, Mice were administered Ad-HO-1 (5X10⁸ pfu) or empty vector (Ad-X-LacZ, 5X10⁸ pfu) for 48 hours, and lung lysates were then immunoblotted against HO-1 or β-tubulin (loading control). c, PAEC stably overexpressing human HO-1 gene (LSN/HO-1) were transfected with different HO-1 siRNA directed against human HO-1 (sequences 1-3) at 300 nM and 600 nM doses for 24-72 hours and immunoblotted against HO-1 or β-tubulin (loading control).

Figure 4. HO-1 siRNA significantly increased while HO-1 overexpression decreased apoptosis in PAEC and mouse lung during A-R and I-R, respectively. a, PAEC were untransfected (control), transfected with nonspecific siRNA (nonspecific siRNA), transfected with HO-1 siRNA directed against rodent siRNA (siRNA), stably transfected with empty retro-viral vector (LXSN), stably transfected with human HO-1 overexpressing retro-viral vector (LSN/HO-1), HO-1 overexpressors transfected with HO-1 siRNA directed against human HO-1 (LSN/HO-1/Human siRNA), or HO-1 overexpressors transfected with HO-1 siRNAs directed against both human and rodent HO-1 (LSN/HO-1/Double siRNA) and then exposed to room air (RA), 24 hours anoxia (24A) or 24 hours anoxia followed by 1 hour reoxygenation (A-R). Apoptosis was quantitated by FACS analysis and is graphically represented. Data represent the mean of 3 independent experiments ± S.E.M. *P<0.05, compared to corresponding siRNA 24A and A-R. #P<0.05, compared to corresponding LSN/HO-1/Human siRNA 24A and A-R. b, Mice were untreated (naive, panel A) or subjected to lung I-R after no treatment (I-R, panel B), intranasal nonspecific siRNA at 2 mg/kg (nonspecific siRNA/I-R, panel C), intranasal HO-1 siRNA directed against rodent HO-1 (HO-1 siRNA/I-R, panel D), intranasal empty adenoviral vector at 5x10⁸ pfu (Ad-X-LacZ/I-R, panel E) or intranasal adenoviral-HO-1 (Ad-HO-1/I-R, panel F). Lung sections were then processed for TUNEL staining.
(Arrows indicate representative TUNEL-positive cells). c, Graphical quantitation of TUNEL positive cells in mouse lungs from the conditions shown in 4b. Data represent the mean of 3 independent experiments ± S.E.M. * P<0.05, compared to naïve, # P<0.05, compared to I-R.

**Figure 5.** HO-1 siRNA increased while HO-1 overexpression decreased Fas expression and caspase 3 activity in PAEC during A-R. PAEC were untransfected (control), transfected with nonspecific siRNA (nonspecific siRNA), transfected with HO-1 siRNA directed against rodent siRNA (siRNA), stably transfected with empty retro-viral vector (LXSN), stably transfected with human HO-1 overexpressing retro-viral vector (LSN/HO-1), HO-1 overexpressors transfected with HO-1 siRNA directed against human HO-1 (LSN/HO-1/Human siRNA), or HO-1 overexpressors transfected with HO-1 siRNAs directed against both human and rodent HO-1 (LSN/HO-1/Double siRNA) and then exposed to room air (RA), 24 hours anoxia (24A) or 24 hours anoxia followed by 1 hour reoxygenation (A-R). a, PAEC were stained with anti-Fas or anti-rat IgG (Negative Control) antibody during A-R and Fas expression was detected by FACS analysis. The data are representative of three independent experiments. b, Graphical quantitation of the percentage of total cells that express Fas during the various conditions. The data represent the mean of three independent experiments ± S.E.M. * P<0.05, compared to corresponding siRNA 24A and A-R. #P<0.05, compared to corresponding LSN/HO-1/Human siRNA 24A and A-R. c, Caspase 3 activity in PAEC was detected during A-R and is graphically represented. Data represent the mean of 3 independent experiments ± S.E.M. * P<0.05, compared to corresponding siRNA 24A and A-R. # P<0.05, compared to corresponding LSN/HO-1/Human siRNA 24A and A-R.
**Figure 1a**

|    | RA | A-R | 1 | 2 | 3 | 4 | 5 | HO-1 siRNA/A-R |
|----|----|-----|---|---|---|---|---|----------------|
| HO-1 |    |     |   |   |   |   |   | HO-1 siRNA sequence |
| β-tubulin |    |     |   |   |   |   |   |                |
Figure 1b

Upper Panel

|    | RA 1 | A-R 2 | HO-1 siRNA/A-R |
|----|------|-------|----------------|
|    | 600nM|       | 3              |
|    | 300nM|       | 4              |
|    | 100nM|       | 5              |

Lower Panel

|    | RA 1 | A-R 2 | Nonspecific siRNA/A-R |
|----|------|-------|------------------------|
|    | 600nM|       | 3                      |
|    | 300nM|       | 4                      |
|    | 100nM|       | 5                      |

HO-1
β-tubulin
Figure 1c

Control

siRNA-Biotin
Figure 2a

| Naïve  | I-R   | 4h | 8h | 16h |
|--------|-------|----|----|-----|
| 1      | 2     | 3  | 4  | 5   |

siRNA/I-R

- HO-1
- β-tubulin
Figure 2b

|       | Naïve | I-R  | siRNA | Nonspecific siRNA |
|-------|-------|------|-------|-------------------|
| Lung  | 1     | 2    | 3     | 4                |
|       |       |      |       |                   |
| Lung  |       |      |       |                   |
|       |       |      |       |                   |
| Kidney|       |      |       |                   |
|       |       |      |       |                   |
| Liver |       |      |       |                   |
|       |       |      |       |                   |
| Heart |       |      |       |                   |
|       |       |      |       |                   |

**Lung**
- HO-1
- β-tubulin

**Kidney**
- HO-1
- β-tubulin

**Liver**
- HO-1
- β-tubulin

**Heart**
- HO-1
- β-tubulin
Figure 2c

| Naïve | 4h | 16h |
|-------|----|-----|
| HO-1 siRNA |

A  B  C
Figure 3a

| LXSN | LSN/HO-1 |
|------|----------|
| ![Image of LXSN](#) | ![Image of LSN/HO-1](#) |

- **HO-1**
- **β-tubulin**
Figure 3b

Upper Panel

|        | Naïve | 48h | 72h | 5d | 7d |
|--------|-------|-----|-----|----|----|
| 1      |       |     |     |    |    |
| 2      |       |     |     |    |    |
| 3      |       |     |     |    |    |
| 4      |       |     |     |    |    |
| 5      |       |     |     |    |    |

Ad-HO-1

HO-1

β-tubulin

Lower Panel

|        | Naïve | 48h | Ad-HO-1 | Ad-X-LacZ |
|--------|-------|-----|---------|-----------|
| 1      |       |     | 48h     | 48h       |
| 2      |       |     |         |           |
| 3      |       |     |         |           |

HO-1

β-tubulin
Figure 3c

|        | LXSN | LSN/HO-1 | Sequence 1 300nM | Sequence 1 600nM | Sequence 2 300nM | Sequence 2 600nM | Sequence 3 300nM | Sequence 3 600nM |
|--------|------|----------|------------------|------------------|------------------|------------------|------------------|------------------|
| 24h    |      |          |                  |                  |                  |                  |                  |                  |
| 48h    |      |          |                  |                  |                  |                  |                  |                  |
| 72h    |      |          |                  |                  |                  |                  |                  |                  |

- **24h**: HO-1
- **48h**: β-tubulin
- **72h**: HO-1
Figure 4a

This figure illustrates the percentage of apoptotic cells under different conditions. The x-axis represents different treatments: Control, Nonspecific siRNA, siRNA, LXSN, LSN/HO-1, LSN/HO-1/Human siRNA, and LSN/HO-1/Double siRNA. The y-axis represents the percentage of apoptotic cells.

- Control: Treatment 1
- Nonspecific siRNA: Treatment 2
- siRNA: Treatment 3
- LXSN: Treatment 4
- LSN/HO-1: Treatment 5
- LSN/HO-1/Human siRNA: Treatment 6
- LSN/HO-1/Double siRNA: Treatment 7

Each bar represents the percentage of apoptotic cells with standard error. Significant differences are indicated by asterisks (*) and number signs (#).
Figure 4c

- Naïve
- I-R
- Nonspecific siRNA
- siRNA
- Ad-X-LacZ
- Ad-HO-1

0 5 10 15 20 25 30 35

Apoptotic Cells (%)

1 2 3 4 5 6
Figure 5b

- Fas Expression (%)

| Treatment                      | RA   | 24A  | A-R  |
|--------------------------------|------|------|------|
| Control                        |      |      |      |
| Nonspecific siRNA              |      |      |      |
| siRNA                          |      |      |      |
| LXSN                           |      |      |      |
| LSN/HO-1                       |      |      |      |
| LSN/HO-1/Human siRNA           |      |      |      |
| LSN/HO-1/Double siRNA          |      |      |      |

- Significance:
  - *: p < 0.05
  - #: p < 0.01
Figure 5c

Caspase 3 activity (Fold Induction)

- Control
- Nonspecific siRNA
- siRNA
- LXSN
- LSN/HO-1
- LSN/HO-1/Double siRNA

Legend:
- RA
- 24A
- A-R