Transactivation of Inducible Nitric Oxide Synthase Gene by Kruppel-like Factor 6 Regulates Apoptosis during Influenza A Virus Infection

Victoria Mgbemena, Jesus A. Segovia, Te-Hung Chang, Su-Yu Tsai, Garry T. Cole, Chiung-Yu Hung and Santanu Bose

*J Immunol* 2012; 189:606-615; Prepublished online 18 June 2012; doi: 10.4049/jimmunol.1102742
http://www.jimmunol.org/content/189/2/606
Transactivation of Inducible Nitric Oxide Synthase Gene by Kruppel-like Factor 6 Regulates Apoptosis during Influenza A Virus Infection

Victoria Mgbemena,*† Jesus A. Segovia,*† Te-Hung Chang,* Su-Yu Tsai,* Garry T. Cole,†‡ Chiung-Yu Hung,†‡ and Santanu Bose*

Influenza A virus (flu) is a respiratory tract pathogen causing high morbidity and mortality among the human population. NO is a cellular mediator involved in tissue damage through its apoptosis of target cells and resulting enhancement of local inflammation. Inducible NO synthase (iNOS) is involved in the production of NO following infection. Although NO is a key player in the development of exaggerated lung disease during flu infection, the underlying mechanism, including the role of NO in apoptosis during infection, has not been reported. Similarly, the mechanism of iNOS gene induction during flu infection is not well defined in terms of the host transactivator(s) required for iNOS gene expression. In the current study, we identified Kruppel-like factor 6 (KLF6) as a critical transcription factor essential for iNOS gene expression during flu infection. We also underscored the requirement for iNOS in inducing apoptosis during infection. KLF6 gene silencing in human lung epithelial cells resulted in the drastic loss of NO production, iNOS promoter-specific luciferase activity, and expression of iNOS mRNA following flu infection. Chromatin immunoprecipitation assay revealed a direct interaction of KLF6 with iNOS promoter during in vitro and in vivo flu infection of human lung cells and mouse respiratory tract, respectively. A significant reduction in flu-mediated apoptosis was noted in KLF6-silenced cells, cells treated with iNOS inhibitor, and primary murine macrophages derived from iNOS knockout mice. A similar reduction in apoptosis was noted in the lungs following intratracheal flu infection of iNOS knockout mice. The Journal of Immunology, 2012, 189: 606–615.

Influenza A virus (flu) is an ssRNA virus that causes severe respiratory diseases upon infection of the airway (1, 2). Flu infection among high-risk individuals (e.g., elderly, immunocompromised individuals) manifests itself in the progression of an inflammatory disease state such as pneumonia (1–3).

NO (4) plays an important role in the innate immune response Against pathogens (4–9). Although it confers a protective role in the infected host, it can also exacerbate the disease pathology associated with infection. This detrimental effect of NO is due to its ability to launch a hyperinflammatory response, leading to tissue damage (10). Two mechanisms are primarily responsible for inducing NO-mediated tissue damage: action of NO-induced inflammatory mediators and apoptosis of NO-targeted cells. With regard to host defense against viruses, NO could act as a “double-edged sword.” Although functioning as an antiviral molecule, NO can contribute to the progression/exaggeration of virus-induced disease (11). Flu infection results in NO production as a result of induction of the inducible NO synthase (iNOS) gene (12). Although a specific transcription factor required for iNOS gene expression during flu infection has not been identified and characterized, one study suggested that NF-kB (an inflammatory transcription factor) may play a role in iNOS gene activation during flu infection (12). NO produced from the lungs during infection contributes to tissue damage and enhanced disease pathology associated with flu infection (13–15). NO-mediated apoptosis represents one of the key factors contributing to enhanced inflammation and tissue damage during noninfectious respiratory diseases, such as asthma and chronic obstructive pulmonary disease (10, 16). Although flu infection results in NO production, the contribution of NO in apoptosis during infection is not known. Furthermore, with the exception of NF-kB, the role of noninflammatory transcription factor(s) in the regulation of iNOS gene expression during respiratory virus infection (especially during flu infection) has not been examined.

In the current study, we identified triple zinc finger containing DNA binding transcription factor Kruppel-like factor (KLF6 (17, 18) as a transcription factor required for iNOS gene induction during flu infection. We also demonstrated that induction of iNOS (leading to production of NO) during flu infection contributes to apoptosis of
both epithelial and immune cells (i.e., macrophages). The in vivo physiological relevance of the contribution of iNOS to apoptosis during flu infection was further documented using wild-type (WT) and iNOS knockout (KO) mice.

The KLF family of transcription factors controls a wide spectrum of biological and physiological processes, including cell growth, cell proliferation, and differentiation (17, 18). Although KLF factors have been implicated in regulating normal cellular/tissue homeostasis, their role during infection has not been examined. In the current study, we identified KLF6 as a regulator of iNOS gene expression during flu infection; furthermore, we demonstrated the requirement of iNOS expression for apoptosis during flu infection.

Materials and Methods

Virus and cell culture

Influenza A (A/PR/8/34 (H1N1) virus (flu) was grown in the allantoic cavities of 10- to 14-day embryonated eggs (19). Virus was purified by centrifugation (two times) on discontinuous sucrose gradients (20). A549 cells were maintained in DMEM supplemented with 10% FBS, penicillin, streptomycin, and glutamine (21). Bone marrow-derived macrophages (BMDMs) were obtained from femurs and tibias of WT and iNOS KO mice and were cultured for 6–8 d, as described earlier (19, 22). Cells were plated on six-well plates containing RPMI 1640, 10% FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin, and 20 ng/mL GM-CSF. The flu titer was monitored by plaque-assay analysis with MDCK cells.

Generation of stable short hairpin RNA-expressing A549 cells

A549 cells transduced (in the presence of 5 μg/mL polybrene at a multiplicity of infection [MOI] of lentivirus) with either scrambled short hairpin RNA (shRNA) (control) or KLF6-specific shRNA-expressing lentivirus (Santa Cruz Biotechnology, Santa Cruz, CA) were selected in the presence of puromycin dihydrochloride. Antibiotic-resistant cells were expanded to generate a population of stable cells expressing either scrambled shRNA or KLF6-specific shRNA.

RT-PCR

Total RNA was extracted from A549 cells using TRIzol reagent (Invitrogen). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was performed using 0.25 U Taq polymerase, 10 pmol each oligonucleotide primer, 1 mM MgCl2, and 100 μM deoxy- nucleotide triphosphates in a final reaction volume of 25 μL. Following amplification, the PCR products were analyzed on 1.5% agarose gel. Equal loading in each well was confirmed by analyzing the expression of the housekeeping gene GAPDH. The following primers were used to detect the indicated genes by RT-PCR: GAPDH forward, 5′-GTCAGTTGGTG-GACCTGGACCT-3′; GAPDH reverse, 5′-AGGCTGCTACATGGCAACTG-3′; Human iNOS forward, 5′-TCCAGGAGCAACAGCACCATCCA-3′; iNOS reverse, 5′-GAGTTGGCCGTGTGGTATG-3′; Human KLF6 forward, 5′-CTCTCGACCTGGAAGCTTATGCTAC-3′; and KLF6 reverse, 5′-ACAGCTCGAGGAACTTTCTCCC-3′.

Chromatin immunoprecipitation assay

The standard chromatin immunoprecipitation (ChIP) assay protocol (23) was followed for examining the binding of KLF6 to iNOS promoter in cells and lung tissue. Following chromatin isolation from (at 1 MOI) human A549 cells and mouse BMDMs (after cross-linking with formaldehyde), it was immunoprecipitated at 4°C with either human or mouse specific anti-KLF6 Ab or isotype–matched control IgG (Santa Cruz Biotechnology). Human cell and mouse iNOS promoter-specific PCR primer pairs (encompassing the consensus CACCCC KLF6 binding site located in the proximal region of the iNOS promoter) were used to analyze the Ab-precipitated DNA. As a negative control, PCR primer pairs corresponding to the iNOS promoter region that does not possess a consensus KLF6 binding site were used. Primers used for the ChIP assay are shown in Table 1. Lung tissue derived from WT and iNOS KO mice was also used for ChIP analysis, as described previously (23). To demonstrate that the amount of DNA used for immunoprecipitation (with either KLF6 Ab or control Ab) was equivalent for each experiment, an equal portion of input DNA was analyzed for each sample.

Viral infection of cells

A549 cells were infected with purified flu in reduced serum and antibiotic-free Opti-MEM medium (Life Technologies). Following adsorption for 1.5 h at 37°C, cells were washed twice with serum-containing DMEM, and the infection was continued in the presence of serum-containing DMEM for the specified times. BMDMs were grown in RPMI 1640 medium supplemented with 10% FBS, sodium pyruvate, l-glutamine, GM-CSF, HEPES buffer. BMDMs were infected with purified flu in serum and antibiotic-free RPMI 1640 medium. Following adsorption for 1.5 h at 37°C, cells were washed twice with serum-containing RPMI 1640, and the infection was continued in the presence of serum-containing RPMI 1640 for the specified times. For some experiments, A549 cells were pretreated with all-equal water (vehicle) or N6-(L-argininyl)-5-lysine dihydrochloride (L-NIL) (200 μM; Cayman Chemical) for 2 h before infection. Infection was continued in the presence of L-NIL.

NO quantification

NO production from infected cells was determined by measuring nitrite levels in the medium supernatant, as described previously (24). Medium supernatant collected from mock- and flu-infected cells were mixed with Griess reagent for 10 min at room temperature. The absorbance (at 543 nm) was measured using a microplate reader. Diluted sodium nitrite was used to derive a standard curve.

Luciferase assay

Luciferase assay was performed, as described previously (19, 25). A549 cells were transfected (using Lipofectamine 2000 from Invitrogen) with pRL-null-Renilla luciferase and luciferase (firefly) reporter gene fused to human iNOS promoter (iNOS-luc). At 24 h posttransfection, cells were infected with flu. At 12 and 24 h postinfection, cells were washed once with PBS and then lysed using passive lysis buffer (Promega). Luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer’s protocol. Transfection efficiency was normalized by measuring expression of Renilla luciferase. Luciferase units were measured by standard methodology and are presented as fold activation of luciferase activity.

Apoptosis assay

Flu-infected A549 cells and BMDMs were examined for apoptosis by annexin V labeling, using an annexin V/propidium iodide (PI) apoptosis detection kit (BioVision, Milpitas, CA) (26, 27).

Flu infection of mice

Six- to eight-wk-old pathogen-free WT C57BL/6 and iNOS KO C57BL/6 mice (The Jackson Laboratory) were anesthetized and inoculated (via intratracheal route) with flu (5 × 106 PFU/mouse) in 100 μl Opti-MEM medium (Invitrogen) (19). Mock-infected animals were sham inoculated with 100 μl Opti-MEM. At 5 d postinfection, whole lung was collected. Lungs were used for in situ TUNEL analysis (with lung tissue sections) and Western blot analysis (with lung homogenate) with anti–caspase-3 Ab (Cell Signaling Technology). Another set of WT mice was infected with flu for 5 d, and the isolated lungs were used for tissue ChIP assay. The mouse experiments performed in the current study were reviewed and approved by The University of Texas Health Science Center at San Antonio (UTHSCSA) Institutional Animal Care and Use Committee.

In situ TUNEL assay

TUNEL assay was performed to evaluate apoptotic cell death in the respiratory tract. Formalin-fixed lungs from flu-infected WT and iNOS KO mice were used for in situ TUNEL assay using an ApopTag Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA). Light microscopy was performed to capture digital images of the TUNEL-stained lung sections. The digital images were used to count the number of TUNEL+ cells in the lung sections using ImageJ software from the National Institutes of Health (http://rsbweb.nih.gov/ij/), as described previously (28). For each analysis, an 85.89 × 64.74 μm area of TUNEL-stained lung section was scanned by ImageJ software. Gross apoptotic area was expressed as pixels/μm, and this value was used to calculate the percentage apoptotic area during each analysis. Three mice from each group were used to prepare lung sections (i.e., three flu-infected WT mice and three flu-infected iNOS KO mice). Data were collected from 27 areas (9 areas/mouse) of the lung sections from each experimental group (i.e., 27 areas for flu-infected WT mice and 27 areas for flu-infected iNOS KO mice). The values obtained from 27 lung section areas of each experimental group (WT and iNOS KO) were used for statistical analysis.
**Results**

**KLF6 is required for NO production**

To study the role of KLF6 during infection, we used KLF6-specific shRNA to silence KLF6 expression in human lung epithelial A549 cells. A549 cells are routinely used as model type II human alveolar epithelial cells, and alveolar cells are a major target of flu during productive infection of the human respiratory tract. A lentivirus-mediated shRNA delivery system was used to generate stable A549 cells lacking KLF6 expression. Loss of KLF6 is evident in stable cells expressing KLF6-specific shRNA (Fig. 1A). Control cells represent stable cells derived after transduction with scrambled shRNA expressing lentivirus.

Control and KLF6-silenced cells were subsequently used to examine whether KLF6 plays any role in NO production or iNOS gene expression during flu infection. To assess the requirement of KLF6 for NO production, control and KLF6-silenced cells were infected with flu. At 12 h postinfection, the medium supernatant was collected to measure nitrite (the end product of NO) by Griess reagent. Griess assay revealed a significant reduction in NO production from KLF6-silenced cells compared with control cells (Fig. 1B). These results demonstrated that KLF6 is required for NO production during flu infection.

**KLF6 is required for iNOS gene expression**

To further evaluate the mechanism responsible for KLF6-dependent NO production, we investigated whether KLF6 regulates iNOS gene expression. Total RNA was isolated from control and KLF6-silenced cells infected with flu for 12 h. The RNA was used to determine iNOS mRNA levels by RT-PCR. iNOS gene expression is regulated by KLF6 at the transcriptional level, because low levels of iNOS transcripts were detected in flu-infected KLF6-silenced cells compared with infected control cells (Fig. 1C).

The loss of iNOS mRNAs in KLF6-silenced cells suggested that KLF6 could positively transactivate the iNOS promoter. To test this possibility, we used luciferase reporter gene fused to human iNOS promoter (iNOS-luc). Control and KLF6-silenced cells transfected with luciferase-expressing plasmids were infected with flu. Expression of the luciferase gene was monitored at 12 and 24 h postinfection. KLF6 directly regulates the iNOS promoter during flu infection, because a significant reduction in luciferase activity was observed in cells lacking KLF6 expression (Fig. 1D). These results showed that KLF6 is an important transcription factor regulating iNOS gene expression during flu infection.

**Binding of KLF6 to iNOS promoter during infection of human lung epithelial cells**

The role of KLF6 in the transactivation of the iNOS gene was further confirmed by performing a ChIP assay to investigate the direct association of KLF6 with iNOS promoter during infection. Human lung epithelial A549 cells were either mock infected or infected with flu for 6, 12, or 24 h. At various times postinfection, chromatin from these cells was immunoprecipitated using either isotype-matched control IgG (negative control) or anti-KLF6 Ab. The KLF6–DNA binding complex was analyzed by PCR using primers corresponding to the KLF6 binding site on the human iNOS promoter (29) (Table I). A primer specific for the non-KLF6–binding region on the human iNOS promoter element (an upstream region in the human iNOS gene devoid of the KLF6-responsive region) (Table I) was used as a negative control. Although KLF6 binding to the human iNOS gene was not detected in mock-infected cells, flu infection led to the association of KLF6 with the human iNOS promoter (Fig. 2). Interestingly, maximal KLF6 binding was detected at 12 h postinfection, which corresponds to the time at which iNOS induction and NO production were observed during infection of A549 cells (Fig. 1). The specificity of KLF6 binding to the human iNOS promoter is borne out by the observation that immunoprecipitation with control IgG did not generate any amplified product. Moreover, amplification with primers that do not correspond to the KLF6 binding site on the human iNOS promoter did not yield any amplified product. These results demonstrate that, during flu infection, KLF6 directly transactivates human iNOS gene expression following its association with the human iNOS promoter.

**KLF6 regulates apoptosis during infection**

Our studies uncovered an important function for KLF6 during flu infection, because it is required for iNOS expression and subsequent NO production from infected cells. Previous studies showed that NO is a contributing factor for exaggerated lung disease (due to lung tissue damage) during flu infection (12–15). Because apoptosis could induce tissue damage, we next investigated whether the KLF6 gene is required for apoptosis during infection. Control and KLF6-silenced cells were infected with flu (0.5 MOI) for 48 h. Postinfection, the cells were used to determine apoptosis by annexin V staining. Annexin V staining (which signifies early apoptosis) revealed a significant reduction in the apoptosis of infected KLF6-silenced cells compared with control cells (Fig. 3). These results demonstrated that KLF6 could directly regulate important cellular activity (i.e., programmed cell death or apoptosis) during flu infection.

**iNOS is required for apoptosis during infection of epithelial cells and macrophages**

Based on our results showing the requirement of KLF6 for optimal apoptosis and transactivation of the iNOS gene by KLF6, we...
speculated that iNOS (by generating and producing NO) may contribute to apoptosis during flu infection. No study has evaluated the role of NO (and iNOS) in apoptosis during flu infection. The role of iNOS was examined by inhibiting iNOS enzymatic activity with the specific iNOS inhibitor L-NIL (30–33). A549 cells were treated with either vehicle (water) or L-NIL (200 mM) during flu (0.5 MOI) infection. A similar concentration of L-NIL (100–300 μM) was used previously to inhibit iNOS activity in lung epithelial cells (including A549 cells) (30–33). We and other investigators (30–33) did not observe any cytotoxicity in lung epithelial cells treated with either vehicle (water) or L-NIL (300 μM) during flu (0.5 MOI) infection.

### Table I. ChIP primers

<table>
<thead>
<tr>
<th>Product</th>
<th>Optimal Annealing Temperature (˚C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human iNOS reverse: 5'-GAGACTTGTTCATCTTGGCTAGCTC-3'</td>
<td>321 bp (encompassing −45 to −366 region of human iNOS promoter)</td>
</tr>
<tr>
<td>Human iNOS forward: 5'-GGAGACCTCCTTGCTGGAGGGA-3'</td>
<td>249 bp (encompassing −4417 to −4666 region of human iNOS promoter)</td>
</tr>
<tr>
<td>Human iNOS forward: 5'-ACCACTAAGGAAOCAGGACACAG-3'</td>
<td>229 bp (encompassing −12 to −241 region of mouse iNOS promoter)</td>
</tr>
<tr>
<td>Mouse iNOS reverse: 5'-GCTCTTGTTTGGCCTGCAGAAG-3'</td>
<td>320 bp (encompassing −1157 to −1477 region of mouse iNOS promoter)</td>
</tr>
</tbody>
</table>

FIGURE 2. Binding of KLF6 to human iNOS promoter during flu infection. ChIP assay on A549 cells that were either mock infected or infected with flu for 6, 12, or 24 h. Both anti-KLF6 Ab (control Ab) and isotype-matched control Ab (control Ab) were used. An upstream region in the human iNOS gene devoid of the KLF6 responsive region was probed as a negative control. All ChIP data are representative of three independent experiments. input, Input DNA for each experiment.

FIGURE 3. KLF6 expression is required for optimal apoptosis following flu infection. (A) Percentage of apoptotic (annexin V+) cells in flu-infected control (expressing scrambled shRNA) and KLF6-silenced (expressing scrambled shRNA) A549 cells. Apoptosis was quantified using an annexin V staining assay. Values represent mean ± SEM from five independent experiments performed in triplicate. The values in (A) were used to calculate the percentage of apoptosis in flu-infected control and KLF6-silenced cells. *p < 0.05, two-tailed t test.
epithelial cells treated with L-NIL (100–200 μM) for 48 h. At 48 h postinfection, the apoptosis status of infected cells was examined by annexin V staining (annexin V+ and PI− cells represent apoptotic cells). Inhibition of iNOS activity markedly diminished the apoptosis of flu-infected cells (Fig. 4A, 4B). A nearly 80% reduction in apoptosis was observed in L-NIL–treated cells compared with control cells. To our knowledge, these results uncovered, for the first time, the role of NO in inducing apoptosis during flu infection. NO specifically contributes to apoptosis during flu infection, because iNOS inhibition did not significantly alter the necrotic status (annexin V− and PI+ cells) of infected cells (Fig. 4C).

The role of iNOS in regulating apoptosis was further confirmed using primary BMDMs isolated from iNOS KO mice. WT and iNOS KO BMDMs were infected with flu (0.5 MOI) for 48 h. Postinfection, the apoptotic status was evaluated by annexin V staining. As shown in Fig. 5A and 5B, a significant decrease in apoptosis (36%) was observed in flu-infected iNOS KO BMDMs compared with WT BMDMs. These results once again confirmed that iNOS/NO plays a crucial role in apoptosis induction during flu infection. In contrast, the necrotic status of cells remained unchanged in flu-infected WT BMDMs versus iNOS KO BMDMs (Fig. 5C).

**Binding of KLF6 to iNOS promoter during infection of primary macrophages**

Experiments performed with WT and iNOS KO primary BMDMs revealed a role for iNOS in apoptosis during flu infection (Fig. 5). Because KLF6 regulates iNOS expression, we next investigated whether, similarly to lung epithelial cells, KLF6 also associates with the iNOS promoter during flu infection of primary mouse macrophages. Previous studies mapped the KLF6 binding site in the human iNOS gene to the proximal region (position −91 to −95) in the mouse iNOS gene. We identified a similar proximal consensus CACCC sequence (at position −164 to −168) (29). The consensus CACCC sequence proximal to the mouse iNOS gene was critical for KLF6 binding to the human iNOS gene. We identified a similar proximal consensus CACCC sequence (at position −91 to −95) in the mouse iNOS gene.

BMDMs were either mock infected or infected with flu for 6, 12, or 24 h. Chromatin from the BMDMs was immunoprecipitated using either an isotype-matched control IgG (negative control) or anti-KLF6 Ab. A primer corresponding to the KLF6 binding site on the mouse iNOS promoter was used to analyze the KLF6–DNA binding complex by PCR. A primer specific for the non-KLF6–binding region on the mouse iNOS promoter (an upstream region in the mouse iNOS gene) was used as a negative control. Although KLF6 binding to the mouse iNOS gene was not observed in mock-infected BMDMs, flu infection resulted in recruitment of KLF6 to the mouse iNOS promoter (Fig. 6). Interestingly, KLF6 association was detected early during infection (i.e., 6–12 h postinfection), and the association of KLF6 with the iNOS promoter was lost at 24 h postinfection (Fig. 6). The time frame for KLF6 interaction with the mouse iNOS promoter corresponded with NO production from flu-infected BMDMs, because optimal NO production from flu-infected BMDMs was observed at 6–12 h post-infection (data not shown). The specificity of KLF6 binding to the mouse iNOS promoter is borne out by the observation that amplification with nonspecific primers (i.e., primers that do not correspond to the KLF6 binding site on the mouse iNOS promoter) did not yield any amplified product. Moreover, immunoprecipitation with control IgG did not generate any amplified product (data not shown). These results demonstrated that KLF6 interacts with the mouse iNOS promoter during flu infection of primary macrophages.

**iNOS expression is essential for optimal apoptosis in the flu-infected respiratory tract**

The in vivo physiological role of iNOS was evaluated by infecting iNOS KO mice with flu. Previous studies showed that, compared with WT mice, iNOS KO mice exhibit reduced lung disease severity following flu infection (13–15). Interestingly, the reduced lung disease was not due to diminished virus replication (infection), because flu burden in the lungs of WT and iNOS KO mice was similar. In contrast with previous reports, our study revealed...
that flu replication (as assessed by flu hemagglutinin expression in the lungs) is similar in WT and iNOS KO mice (Supplemental Fig. 1). Because apoptosis contributes significantly to disease severity, reduced apoptosis in flu-infected iNOS KO mice may be one of the contributing factors responsible for diminished airway damage in iNOS KO animals. To assess this possibility, WT and iNOS KO mice were inoculated with flu (5 \times 10^4 PFU/mouse) via intratracheal route. At 5 d postinfection, the lungs were collected, and in situ TUNEL assay with lung sections was conducted to evaluate the apoptotic status of the respiratory tract. Diminished apoptosis was observed in the lungs of iNOS KO animals compared with WT mice (Fig. 7A). Scanning of the lung sections for TUNEL+ cells (apoptotic cells) revealed significantly reduced apoptosis in iNOS KO mice (Fig. 7B). A representative TUNEL-stained lung section from flu-infected WT and iNOS KO mice is shown in Supplemental Fig. 2. It is interesting to note that, consistent with a previous report (13), we also observed markedly reduced inflammation (as deduced by cellularity of the airway) in the lungs of infected iNOS KO animals (Supplemental Fig. 2). The TUNEL results were further confirmed by performing Western blot analysis with anti–caspase-3 Ab, which detects both the full-length (35 kDa) and cleaved product (19 kDa) of caspase-3. Cleavage of procaspase-3 serves as a hallmark of apoptosis. Lung homogenates prepared from flu-infected WT and iNOS KO mice were subjected to Western blot analysis with caspase-3 Ab. Although cleaved caspase-3 was abundant in the lungs of WT mice, a decrease in cleaved caspase-3 product was noticed in iNOS KO lungs (Fig. 7C, 7D). These results showed that iNOS is a critical regulator of lung apoptosis during flu infection.

KLF6 binds to the iNOS promoter during flu infection of mice

Our in vitro study showed that KLF6 is essential for iNOS gene expression by virtue of its binding to the iNOS promoter during flu infection of epithelial cells and macrophages (Figs. 2, 6). The in vivo physiological relevance of this observation was further confirmed by examining the interaction of KLF6 with the iNOS promoter in the lungs of mice infected with flu. Tissue ChIP assay was performed using lung harvested from flu-infected mice. Mice were infected with flu via intratracheal route. At 5 d postinfection, lungs were collected. Lungs isolated from mock-infected and flu-infected mice were processed for ChIP assay. Chromatin from the tissue was immunoprecipitated using either isotype-matched control IgG (negative control) or anti-KLF6 Ab. A primer corresponding to the KLF6 binding site on the mouse iNOS promoter was used to analyze the KLF6–DNA binding complex by PCR. A primer specific for the non-KLF6–binding region on the mouse iNOS promoter (an upstream region in the mouse iNOS gene) was used as a negative control. KLF6 binding to the mouse iNOS promoter was not observed in mock-infected animals (Fig. 8). However, flu infection resulted in binding of KLF6 to the mouse iNOS promoter (Fig. 8). The specificity of KLF6 binding to the mouse iNOS promoter is evident by the observation that amplification with nonspecific primers (i.e., primers that do not correspond to the KLF6 binding site on the mouse iNOS promoter) did not
yield any amplified product. Moreover, immunoprecipitation with control IgG did not generate any amplified product (data not shown). These results demonstrated the association of KLF6 with the mouse iNOS promoter during flu infection of the respiratory tract. A schematic model depicting the role of KLF6 and iNOS is shown in Fig. 9.

**Discussion**

iNOS gene expression is tightly regulated, because NO-mediated tissue damage could lead to enhanced disease phenotype (4–11). Because of NO’s ability to induce apoptosis and enhance inflammation (10, 11, 16), NO produced during virus infection significantly contributes to development of the disease state. These events culminate in an exacerbated disease condition as the result of tissue damage. Respiratory viruses, like influenza A virus, induce iNOS expression to generate NO, which contributes to enhanced disease pathophysiology associated with increased damage to the lung tissue (12–15). Although iNOS plays a critical role in determining the severity of the disease state following flu infection, it is not known whether iNOS/NO is required for apoptosis during flu infection or whether noninflammatory transcription factor(s) (with the exception of NF-κB, which is an inflammatory transcription factor) are required for iNOS gene expression during flu infection. Our studies uncovered two mechanisms associated with NO production during flu infection: we demonstrated that NO produced during infection plays an essential role in inducing apoptosis during infection, and we also identified a noninflammatory transcription factor (i.e., KLF6) as a transactivator of the iNOS gene during infection. Based on our studies, we propose a model (Fig. 9) in which flu infection triggers binding of KLF6 to the iNOS promoter, leading to expression of iNOS gene/protein. NO generated by iNOS is a key contributing factor for induction of the apoptotic pathway during infection.
Apoptosis significantly contributes to pathogenesis and lung injury/disease pathology associated with flu infection (34–39). Apoptosis of lung epithelial cells during infection with respiratory viruses, such as respiratory syncytial virus, results in their sloughing, and these cells aggregate with fibrin, mucin to form plugs associated with airway obstruction, a condition prevalent among respiratory syncytial virus-infected infants and children (40, 41). Apart from airway obstruction, a high degree of apoptosis of both lung epithelial cells and immune cells (e.g., macrophages) during respiratory virus infection results in delayed clearance of dead cells from the airway lumen, which culminates in the induction of necrosis and subsequent inflammation of the airway (42). A similar mechanism involving exaggerated apoptosis in the airway may contribute to the development of severe lung damage (including progression to a disease state such as pneumonia) during infection with highly virulent strains of flu (35–39).

Various mechanisms are involved in the induction of apoptosis following flu infection. Apoptosis during flu infection is mediated by Siva-1 expression, extracellular calcium influx, Bax activation, and production of TRAIL (43–47). NO-induced apoptosis represents one of the key factors contributing to enhanced inflammation and tissue damage (10, 16). Moreover, NO production during infection with respiratory viruses, such as flu, results in exaggerated disease pathology attributed to increased lung inflammation and enhanced tissue damage (13–15). Although flu infection results in NO production (12), the contribution of NO in apoptosis during infection has not been evaluated. Our study showed that NO produced during flu infection plays a critical role in inducing apoptosis. The contribution of NO to apoptosis in the airway was also validated in vivo using flu-infected WT and iNOS KO mice. Additionally, we identified KLF6 as an essential transcription factor required for iNOS gene expression during flu infection.

The 16-kb iNOS gene is regulated by numerous transcription factors, such as NF-κB, STAT-1a, AP-1, Oct-1 (octamer factor), T cell factor 4, STAT-3, NF-AT, and NF-IL6 (4). Among these transcription factors, inflammatory transcription factors (e.g., AP-1, NF-κB, NF-AT, and STAT-1a) play an important role during infection and inflammatory response. NO is produced during flu infection, primarily as the result of iNOS induction (12–15). There has been no report that neuronal NOS is involved in NO generation during infection. Thus, inhibition of neuronal NOS activity in A549 cells did not block NO generation following flu infection (Supplemental Fig. 3). The role of NF-κB in iNOS induction during flu infection has been suggested (12). It was also shown that the iNOS gene is regulated by IRF-1 during flu infection (12). However, direct interaction/binding of NF-κB and IRF-1 to the iNOS promoter during flu infection has not been reported. Moreover, with the exception of NF-κB, no other transcription factors have been implicated in transactivation of the iNOS gene during flu infection. In the current study, we uncovered the role for a noninflammatory transcription factor (i.e., KLF6) in controlling iNOS gene expression during flu infection. Our studies also illuminated the role of the KLF family of transcription factors as an important regulator of gene expression associated with infection. NO production was dependent on infection with viable flu, because a significant reduction in NO levels was observed following infection with UV-irradiated flu (UV-flu) (Supplemental Fig. 4A). Interestingly, replication-incompetent flu (UV-flu) did not completely abrogate NO production, suggesting that viral components from input virus could trigger NO production; however, replication is required to sustain this process. The efficiency of UV inactivation was evident from the lack of flu hemagglutinin expression in cells infected with UV-flu (Supplemental Fig. 4B).

The KLF family of transcription factors controls a wide spectrum of biological and physiological processes, including cell growth, cell proliferation, and differentiation (17). The mammalian KLF family consists of 17 DNA-binding proteins that possess three zinc-fingered motifs. KLF transcription factors regulate the function of various organ systems, including the hematological, digestive, cardiovascular, and respiratory systems; thus, they have been implicated in the development/progression of diseases, such as cancer, metabolic disorders, and cardiovascular and inflammatory diseases (17). Among the KLF family, KLF6 is known to regulate gene expression in various tissues by acting as either a transactivator or a repressor of gene expression. KLF6-regulated genes include E-cadherin (48), TGF-β and TGF-β receptors (49), collagen (50), and urokinase plasminogen activator (51). In addition, KLF6 was identified as a tumor-suppressor gene associated mainly with prostate cancer (52). A role for KLF6 in renal ischemia-reperfusion injury (53) and hepatic fibrosis (49, 50, 54) was reported. iNOS expression is also regulated by KLF6 during severe stress-related pathophysiological conditions, such as heat shock, serum starvation, and hypoxia (29). Although the human iNOS gene contains 10 consensus KLF6-binding motif “CAAAAC,” the proximal 0.63-kb region of the iNOS gene is required for KLF6 binding to the iNOS gene for transactivation (29). Another member of the KLF family, KLF4, also regulates iNOS and endothelial NO synthase expression during inflammation of the neuron and endothelium (55, 56). Although KLF factors have been implicated in regulating normal cellular/tissue homeostasis, their role during infection was not known. In the current study, we identified KLF6 as a regulator of iNOS gene expression during flu infection.

Although the mechanism underlying iNOS gene transactivation by KLF6 is not known, we surprisingly observed that polyinosinic-polycytidylic acid (poly-IC) (a TLR3 ligand)–mediated NO production is dependent on KLF6 expression, because KLF6 silencing drastically inhibited NO production following poly-IC treatment (Supplemental Fig. 4C). Several studies reported that activated TLR3 (and poly-IC treatment) triggers iNOS-mediated NO release (57–60), and our results suggested that KLF6 is required for TLR3-mediated iNOS induction. TLR3 is expressed in lung epithelial cells (and TLR3 is activated by poly-IC in these cells) (61, 62); TLR3 activation during flu infection is not only required for the proinflammatory response in lung epithelial cells, it plays an important role in regulating flu pathogenesis in mice (61, 63–65). Thus, it is plausible that activation of the TLR3 pathway (resulting in activation of NF-κB and MAPK signaling) during flu infection results in KLF6 binding to the iNOS promoter, leading to iNOS gene expression. During this event, the iNOS gene-specific transactivating property of KLF6 could be achieved by several mechanism(s): 1) Signal-specific phosphorylation of several KLFs (e.g., KLF4) regulates their transactivating function. For example,
phosphorylation of KLF4 by MAPK results in recruitment of KLF4 to the TGF-βR promoter and TGF-βR gene expression (66). Because TLR3 activates the MAPK pathway, it is possible that KLF6 phosphorylation promotes KLF6 binding to the iNOS promoter. 2) Interaction of KLFs with proteins, such as Sp1 (67), p53 (68), and NF-κB p65 (69) subunit (following NF-κB activation) dictate their transcriptional activity. A similar scenario may exist during flu infection, whereby, upon NF-κB activation (via TLR3), KLF6 may interact with NF-κB subunits or unidentified cellular proteins. These interactions may play a pivotal role in determining the iNOS gene-specific transcription function of KLF6. 3) Apart from phosphorylation, the transactivating property of KLFs are also known to be regulated by additional posttranslational modifications, such as SUMOylation (70, 71). KLF6 may undergo a similar modification following flu infection. In the future, we will dissect the exact mechanism regulating iNOS gene-specific transactivating function of KLF6 during flu infection and characterize the upstream signaling pathway (and mediators) involved in this process.

Our studies demonstrated that KLF6 binds to the iNOS promoter during flu infection to transactivate iNOS gene expression. The importance of KLF6 during this process is evident from its association with the iNOS promoter during flu infection of human lung epithelial cells, primary mouse macrophages, and mouse lung. KLF6 is also expressed in the respiratory tract (72) (Fig. 8), and KLF4 [both KLF4 and KLF6 belong to group 2 of KLF factors (29)] expression is potentially associated with fibrosis lung. KLF6 is also expressed in the respiratory tract (72) (Fig. 8), and KLF4 [both KLF4 and KLF6 belong to group 2 of KLF factors (29)] expression is potentially associated with fibrosis.

Therefore, we envision that KLF6 may also contribute to exaggerated lung disease during flu infection, because KLF6 regulates the expression of iNOS, which is involved in apoptosis/tissue damage. Further studies are required to investigate the functions of KLF6 in the airway tract by using conditionally knocked out transgenic mouse once they are available, because KLF6 KO mice are embryonic lethal.

Acknowledgments
We thank Dr. Serpi C. Erzurum (Department of Pathobiology, Cleveland Clinic, Cleveland, OH) for providing the iNOS-luciferase reporter plasmid. Drs. Ibtisam Echchaguida and Bandana Chatterjee (Department of Molecular Medicine, UTHSCSA) for excellent suggestions, Dr. Linda Roman (Department of Biochemistry, UTHSCSA) for assisting with the in vitro neuronal NO synthesis assay, and Dr. Benjamin J. Daniel and Karla M. Gorena from the UTHSCSA FACS facility for assistance.

Disclosures
The authors have no financial conflicts of interest.


Supplemental Fig - 1
Supplemental Fig - 2

WT  iNOS KO

Flu infection
Supplemental Fig - 3

A

Nitric oxide (µM)

L-thiocitrulline

-  

+ Flu infection

10 µM  100 µM

B

NO synthesis (µM/minute)

L-thiocitrulline (µM)

0  20  40  60  80  100

60  50  40  30  20  10
Supplemental Figure legends

SUPPLEMENTAL FIGURE 1. Expression of flu hemagglutinin (HA) gene in the lungs of infected wild type (WT) and iNOS knock-out (KO) mice. WT and iNOS KO mice were infected with flu. At 3d post-infection RNA prepared from the lung tissue was utilized to examine flu HA expression by RT-PCR analysis. RT-PCR data is representative of three independent experiments.

SUPPLEMENTAL FIGURE 2. iNOS expression promotes enhanced apoptosis in the lung of flu infected mice. TUNEL staining of lung sections from wild type (WT) and iNOS knock-out (KO) mice infected with flu. The apoptotic nuclei (representing apoptosis) are indicated with red arrows. In accordance with previous reports, control (mock-infected) lung sections from both WT and iNOS KO animals did not show any airway inflammation or apoptosis (data not shown).

SUPPLEMENTAL FIGURE 3. Neuronal NOS (nNOS) activity is not required for nitric oxide (NO) production from flu infected cells. (A) A549 cells were pre-treated with either vehicle (ethanol) or nNOS inhibitor S-methyl L-Thiocitrulline (10 μM or 100 μM) (Cayman Chemical) for 2h. The cells were then infected with flu in the absence (in the presence of ethanol) or presence of nNOS inhibitor S-methyl L-Thiocitrulline (10 μM or 100 μM). At 12h post-infection, NO production was measured by monitoring nitrite formation using Griess reagent. Each value represents the mean ± standard deviation from three independent experiments performed in triplicate. (B) The nNOS inhibitor (L-Thiocitrulline) used in the above experiment is active since it inhibited nNOS activity
in an in vitro NO assay. Nitric oxide formation (hemoglobin capture assay) was measured at 23° C as described (Martásek, P., Miller, R. T., Roman, L. J., Shea, T., and Masters, B. S. S. Methods Enzymol. 301: 70-78, 1999), with the exception that the assay was performed in a buffer containing 50 mM Tris-HCl, pH 7.4, and 100 mM NaCl and the indicated concentration of inhibitor. The arginine concentration was 100 μM, and the nNOS concentration was 0.1 μM. The rate of NO synthesis (μM/minute) was determined using an extinction coefficient of 60 mM⁻¹cm⁻¹ at 401 nm.

SUPPLEMENTAL FIGURE 4. Infectious flu virus particle is required for NO production and KLF6 is required for NO production following poly-IC stimulation. (A) NO production from A549 cells infected with either UV-flu (UV-irradiated flu virus) or control-flu (non UV irradiated flu virus) for 12h. NO was measured by monitoring nitrite production using Griess reagent. Each value represents the mean ± standard deviation from three independent experiments performed in triplicate. *p <0.05 using a Student’s t test. (B) A549 cells were infected with either control-flu or UV-flu. At 24h post-infection, flu HA expression was examined by RT-PCR analysis. RT-PCR data is representative of three independent experiments. (C) NO production from untreated (UT) and poly-IC treated (treated with 0.2 μg/ml of poly-IC for 16h) control and KLF6 silenced cells. NO was measured by monitoring nitrite production using Griess reagent. Each value represents the mean ± standard deviation from three independent experiments performed in triplicate. *p <0.05 using a Student’s t test.