

# RSV Causes HIF-1 $\alpha$ Stabilization via NO Release in Primary Bronchial Epithelial Cells

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**Abstract**—RSV infection is characterized by airway edema. Stabilization of hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is important in both inflammation and edema formation. In this study we evaluated whether RSV induced release of nitric oxide (NO) by bronchial airway epithelial cells leading to the stabilization of HIF-1 $\alpha$  and subsequent transcription of VEGF<sub>165</sub>. Primary human bronchial epithelial cells (HBEpC) were used; cell supernatants were analyzed. Western blot analysis was used for the detection of HIF-1 $\alpha$ . Bronchial airway epithelial monolayer permeability was assessed using electric cell-substrate impedance sensing (ECIS) in real time. There was increased stabilization of HIF-1 $\alpha$  in RSV infected cells. Addition of an NO inhibitor blocked RSV mediated HIF-1 $\alpha$  expression. Antagonism of NO also inhibited VEGF production and HBEpC monolayer permeability. Our results demonstrate that in HBEpC, RSV induced NO causes stabilization of HIF-1 $\alpha$  *in vitro*.

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**KEY WORDS:** edema; VEGF; Carboxy-PTIO; ECIS.

## INTRODUCTION

Respiratory syncytial virus (RSV) is the most common cause of lower respiratory tract infections in children worldwide and is recognized to play a role in the pathogenesis of asthma (1). It causes an estimated 31 bronchiolitis associated hospital admissions per 1,000 per year in children aged <1 year (2). It is also recognized as a major cause of morbidity and mortality in immunodeficient patients (3, 4). RSV is a labile paramyxovirus that produces a characteristic fusion of human cells in tissue culture known as the syncytial effect. Two subtypes, A and B, have been identified. Subtype B is characterized as the asymptomatic strain of the virus that the majority of the population experiences. The more severe clinical illnesses

involve Subtype A strain, which tends to predominate in most outbreaks (5).

RSV affects both the upper and lower respiratory tracts, but is more prevalent in lower respiratory illnesses such as pneumonia and bronchiolitis. Infection results in damage to small and medium sized bronchioles. The mechanism and signaling pathways activated by RSV that result in airway epithelial edema and inflammation are not completely understood. RSV replicates in ciliated epithelial cells of the airway. Submucosal edema is a prominent feature of RSV bronchiolitis and pneumonia (6). It has been assumed that the edema is due to the virus induced epithelial damage that is characteristic of these lesions. Recent studies, however, have demonstrated that cytokines such as vascular endothelial growth factor (VEGF) can also directly alter blood vessel permeability (7). We have previously demonstrated that VEGF

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**Abbreviations:** RSV, Respiratory Syncytial Virus; NO, Nitric Oxide; HIF-1 $\alpha$ , Hypoxia Inducible Factor-1 $\alpha$ ; VEGF, Vascular Endothelial Growth Factor; Carboxy PTIO, Carboxy 2-(4-Carboxyphenyl)-4, 4, 5, 5-tetramethylimidazole-1-oxyl-3-oxide sodium salt.

can also cause permeability changes in airway epithelial and mesothelial cell monolayers (8, 9).

RSV infection causes VEGF release (10). VEGF is one of the many target genes for hypoxia inducible factor 1- $\alpha$  (HIF-1 $\alpha$ ); other target genes include those necessary for glycolysis, angiogenesis, and cytokine release (11). HIF-1 $\alpha$  is a heterodimeric basic-helix-loop-helix-PAS transcription factor consisting of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits. The active HIF-1 complex accumulates in the nucleus, binds to a specific DNA sequence, and enhances the transcription of hypoxia-inducible genes like VEGF. The presence of HIF-1 is mainly determined by the presence or absence of HIF-1 $\alpha$  (12, 13). Both HIF-1 $\alpha$  and HIF-1 $\beta$  seem to be expressed in many cell types at the mRNA level, but on the protein level HIF-1 $\alpha$  is degraded under normoxic conditions, while HIF-1 $\beta$  is constitutively expressed (14). HIF-1 $\alpha$  turnover is regulated by the gene encoding the Von Hippel Lindau (VHL) protein which ubiquitinates HIF-1 $\alpha$  under normal conditions (15). Various *in vivo* assays of acute and chronic inflammation demonstrate a profound reliance on HIF-1 $\alpha$  function for cellular infiltration, edema formation, and tissue destruction caused by granulocytes and macrophages (16). Deletion of VHL causes a hyper-inflammatory response, but loss of the HIF-1 $\alpha$  target VEGF eliminates tissue edema only. HIF-1 $\alpha$  is critical in regulating pathways essential for the maintenance of energy homeostasis in myeloid cell types and functional inactivation of it causes greatly inhibited motility, invasiveness, and adhesion in macrophages (16).

RSV infection enhances the release of nitric oxide (NO), through the activation of the inducible isoform of nitric oxide synthase (iNOS) (17, 18). NO has a stimulatory effect on HIF-1 $\alpha$  stabilization. Several studies have shown that in different cell types, chemically diverse NO donors, transfection of NO synthase or macrophage-derived NO evoked HIF-1 $\alpha$  accumulation and HIF-1 $\alpha$  DNA binding followed by target gene expression (14, 17, 18).

Signaling pathways that promotes HIF-1 $\alpha$  stabilization in response to NO point to a genistein-sensitive phosphorylation cascade (17). In this study, we evaluate whether NO production in RSV infection causes induction of the HIF-1 $\alpha$  and VEGF expression that leads to edema formation.

## MATERIALS AND METHODS

### Viral Stock Preparation

RSV (A2 strain) was obtained from the ATCC (Manassas, VA). Hep-2 cells from ATCC were infected

with a low-input multiplicity of infection (MOI). When infection got advanced, cell supernatants were harvested, cells were disrupted by repeated freeze thawing. Cell-debris was pelleted by low-speed centrifugation. Aliquots of clarified supernatants were frozen at  $-70^{\circ}\text{C}$ . Titers of infectivity of stock viruses were determined by inoculation of serial dilutions into Hep-2 cells and by quantification of plaque formation (10).

### Cells and Cell Culture

Primary human bronchial epithelial cells (HBEPc) purchased from Cell Applications (San Diego, CA) were used in these studies. Serum-free bronchial epithelial growth medium with supplements were purchased from the same provider. The HBEPc cells were grown and sub-cultured according to the supplier's recommendations in 48 well plates. On the day of infection, the culture medium bathing the cells was aspirated and the cultures were inoculated with RSV (MOI of 0.05). After adsorption at  $37^{\circ}\text{C}$  for 90 min, the viral solution was removed. The cells were washed twice with media, and incubated at  $37^{\circ}\text{C}$  for the desired periods of time. The supernatants were collected, clarified by low-speed centrifugation, and stored at  $-70^{\circ}\text{C}$  until analyzed. The cell monolayers were harvested for quantification of HIF-1 $\alpha$ . In some experiments the NO inhibitor, Carboxy-PTIO (Calbiochem, San Diego) was added at a concentration of  $100\ \mu\text{M}$  to wells at time zero and 20 h post infection. The cells viability was checked by Trypan blue dye exclusion 48 h post infection with RSV MOI 0.05 and 0.005. The cell viability was 94.4% (MOI 0.05) and 94.1% (MOI 0.005) in RSV infected cultures. In the control cultures the cell viability was 95.1%.

### NO Estimation

NO production was estimated via assay for nitrite ( $\text{NO}_2$ ) and nitrate ( $\text{NO}_3$ ) in confluent HBEPc monolayers. Epithelial monolayers were infected with RSV as described above. Fifty microliters of supernatants from each sample was assayed for NO production. Experiments were repeated three times with duplicate samples. Briefly, 25 mU of nitrate reductase with  $40\ \mu\text{M}$  NADPH in  $25\ \mu\text{L}$  of buffer were added to the samples and incubated for 1 h at  $25^{\circ}\text{C}$ . The reaction was stopped by the addition of  $25\ \mu\text{L}$  of buffer containing 4 mM  $\beta$ -ketoglutarate, 100 mM ammonium chloride, and 200 mM of glucose-6-phosphate dehydrogenase. After further incubation for 10 min,  $150\ \mu\text{L}$  of Griess reagent (0.1% naphthyl ethylenediamine dihydrochloride-1% sulfonic acid in 5% phosphoric acid mixed in equal volumes) was added to

each sample at room temperature. After thorough mixing for 5 min, optical density was measured at 540 nm and the amount of NO<sub>3</sub>/NO<sub>2</sub> is calculated on the basis of standard curves generated with sodium nitrite.

### HIF-1 $\alpha$ Estimation

HIF-1 $\alpha$  was estimated by Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. HBEPc monolayers were infected with RSV for increasing periods of time, as described above. Some wells received the NO scavenger Carboxy PTIO. Cells were scraped, pelleted, and lysed in Laemmli-buffer (2% [wt/vol] SDS, 6% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, and a trace amount of bromophol blue in 200 mmol/L Tris-HCl, pH 7.5). The samples were immediately heated for 5 min at 100°C. Total cell lysates 60  $\mu$ g per sample were subjected to SDS-PAGE on gels containing 5% (wt/vol) acrylamide under reducing conditions. Separated proteins were transferred to PVDF membranes (Polyvinylidene Difluoride, BioRad). Membrane was blocked overnight with 3% BSA, and proteins were detected using the HIF-1 $\alpha$  antibody (1:125 dilution) (BD Transduction Laboratories, San Diego, Ca) for 1 h, followed by several washes with PBS-Tween. Blots were incubated with sheep antimouse secondary antibodies and final detection was performed by chemiluminescence using ECL kit (Enhanced Chemiluminescence; Amersham Pharmacia Biotech, New Jersey) and subsequent autoluminography by exposure to X-ray films (Eastman Kodak). Densitometry was performed using Quantity One software (BioRad).

### VEGF ELISA

VEGF/VPF levels in RSV infected and noninfected epithelial cell culture supernatants were measured by “sandwich” enzyme immunoassay (Quantikine<sup>®</sup> ELISA, R&D System, Minneapolis, MN) as previously described (19). Briefly, the samples were added to 96 well microtiter plates, which was coated with murine monoclonal antibody to VEGF<sub>165</sub>. The unbound protein was washed three times and an enzyme linked polyclonal antibody specific to VEGF<sub>165</sub> was added. The plate was washed again three times and substrate solution was added to the wells. After 30 min of incubation, stop solution was added to each well. The amount of VEGF was determined by optical density of the samples by comparing the standards at 450 nm using the ELISA reader.

### Real-Time Electric Cell-Substrate Impedance Sensing (ECIS)

The real-time electrical resistance of both the RSV infected and noninfected HBEPc monolayers was measured by ECIS (Applied Biophysics, Troy, NY). Briefly, bronchial epithelial cells were cultured on a small gold electrode (10<sup>-4</sup> cm<sup>2</sup>), and culture media was used as the electrolyte. The total electrical resistance was measured dynamically across the bronchial epithelial cell monolayer. Experiments were conducted on wells that achieve 4000–7000 ohms of steady state resistance. Resistance was expressed by the in-phase voltage (proportional to the resistance), which was normalized to the initial voltage and expressed as a fraction of the normalized value (19, 20). In addition to infecting the cells with RSV, some wells were treated with Carboxy PTIO at a concentration of 100  $\mu$ M at time zero and 20 h after infection.

### Statistical Analysis

The significance of differences between experimental and control groups was tested by ANOVA using Sigma-Stat statistical software. The significance of difference between the two groups was tested by all pair wise multiple comparison procedure (Student-Newman-Keuls method), and  $p < 0.05$  was considered significant.

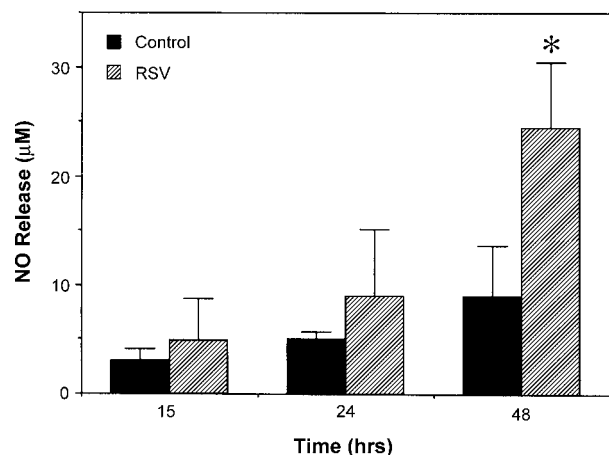
## RESULTS

### RSV Induced NO Production HBEPc *In Vitro*

RSV enhanced NO release in a time dependent manner. NO production by the control cells was 3.1  $\pm$  1.1, 5  $\pm$  0.7, and 9.1  $\pm$  4.6  $\mu$ M following 15, 24, and 48 h incubation, respectively. NO production in RSV infected cells was 4.9  $\pm$  3.9, 9.1  $\pm$  6.1, and 24.5  $\pm$  6  $\mu$ M at 15, 24, and 48 h post infection, respectively (Fig. 1). NO production in RSV infected HBEPc was significantly ( $p < 0.001$ ) increased at 48 h when compared to control.

### RSV Infection Stabilized HIF-1 $\alpha$ in HBEPc *In Vitro*

RSV caused the stabilization of HIF-1 $\alpha$  in HBEPc in a time dependent manner. HIF-1 $\alpha$  was detected at the 24- and 48-h post infection. HIF-1 $\alpha$  expression was higher in HBEPc 48 h post RSV infection. We did not detect any HIF-1 $\alpha$  in the control cells. A significant decrease in HIF-1 $\alpha$  expression was notice in presence of NO inhibitor



**Fig. 1.** RSV mediated NO release in human bronchial epithelial cells. HBEpC were infected with RSV as described in methods and culture supernatants were collected at 15, 24, and 48 h. The data represent mean  $\pm$  SE of three independent experiments. \* $p < 0.01$  compared to control.

(Carboxy-PTIO), suggesting a role of NO in the induction of HIF-1 $\alpha$  in RSV infection (Fig. 2).

#### NO Scavenger Inhibits VEGF Production in HBEpC *In Vitro*

VEGF production was blocked by the NO scavenger carboxy PTIO. Our data showed an increase in VEGF production over time, with a significant increase at 48 h. There was insignificant increase at 24 h, but it significantly increased after 48 h. The VEGF levels in

control were  $430 \pm 13$ ,  $545 \pm 335$ , and  $343 \pm 272$  pg/mL after 6, 24, and 48 h, respectively. The VEGF levels in RSV infected cultures were  $558 \pm 17$ ,  $805 \pm 185$ , and  $1750 \pm 212$  pg/mL at 6, 24, and 48 h, respectively. When the NO scavenger carboxy PTIO was added, the VEGF level were reduced to  $664 \pm 91$ ,  $515 \pm 219$  and  $555 \pm 181$  pg/mL at 6, 24, and 48 h, respectively (Fig. 3).

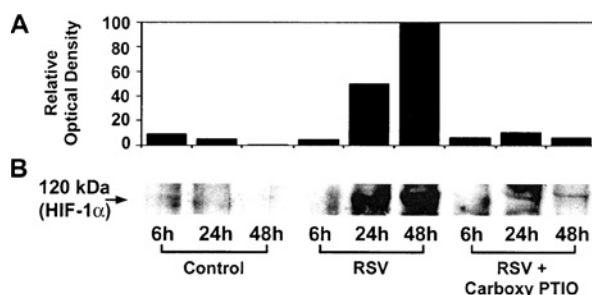
#### NO Scavenger Reversed RSV Induced Permeability in HBEpC Monolayers *In Vitro*

When the cells attach to the gold electrode, the electrical properties of the circuit change. The amplifier measures the resistance across the monolayer (the tightness of the junctions between cells in the monolayer). The uninfected HBEpC cells had resistances between 4500 and 7000 for the healthy uninfected monolayers. After infection with RSV of MOI 0.005 the resistance dropped after 24 h. The decline in resistance was more significant 48 h post infection. Inclusion of carboxy PTIO in RSV infected cultures inhibited HBEpC monolayer resistance indicating a role for NO in bronchial epithelial permeability during RSV infection (Fig. 4).

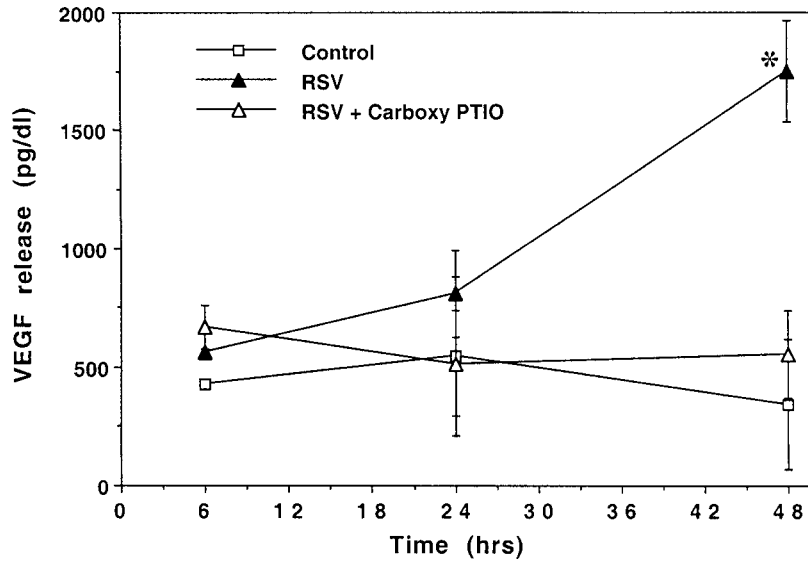
#### DISCUSSION

Our results demonstrate that in RSV infection, HIF-1 $\alpha$  protein was produced in a time dependent manner. The increase in HIF-1 $\alpha$  corresponded temporally to an increase in VEGF production and resistance drop in primary bronchial epithelial cell monolayers. We hypothesized that endogenous NO production, as a result of RSV infection, caused stabilization of HIF-1 $\alpha$  and the subsequent VEGF release and permeability changes across the monolayer. We also demonstrated that by scavenging NO with carboxy PTIO, HIF-1 $\alpha$  protein and VEGF release were decreased to almost control levels. The permeability change of the HBEpC monolayer was reversed despite the presence of RSV infection. We previously demonstrated that VEGF can cause permeability changes in bronchial epithelial monolayers and neutralization of VEGF reversed these changes (8). Our suggested signaling pathway did not appear to involve significant cell toxicity because the changes in permeability and VEGF production were not associated with HBEpC cell death due to RSV infection.

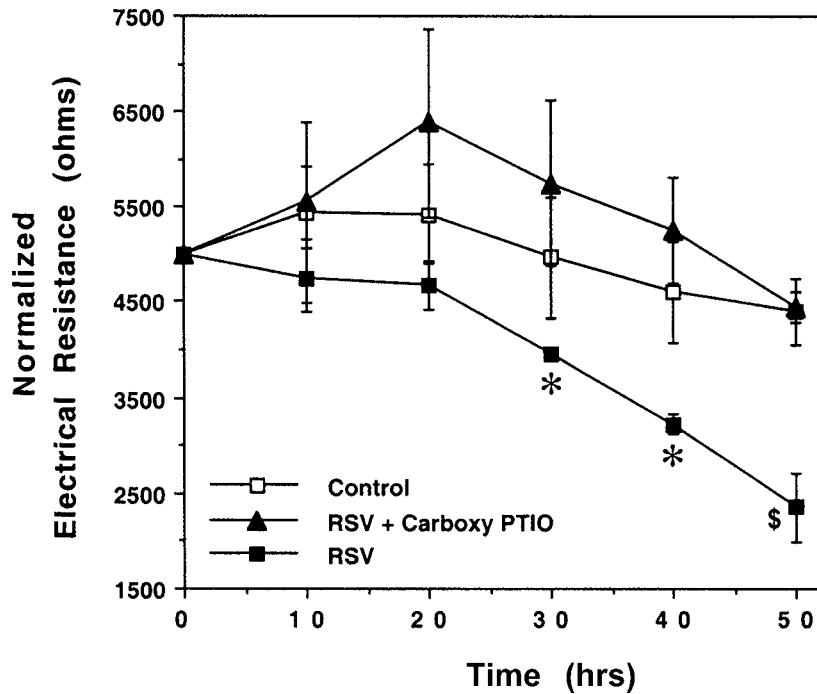
Airway inflammation is an important feature of RSV infection. In RSV infection, plasma and inflammatory cells move into the interstitium, the airway walls and into



**Fig. 2.** RSV infection stabilizes HIF-1 $\alpha$  in human bronchial epithelial cells. HBEpC were infected with RSV (MOI of 0.05) and subjected to Western blot analysis as described in methods. Plate A: Relative optical densities of HIF-1 $\alpha$ ; Panel B: Representative western blot autoradiograph. The Western blot showed the presence of HIF-1 $\alpha$  at 24 and 48 h post infection. Inclusion of Carboxy PTIO blocked the HIF-1 $\alpha$  expression. This is a representative of three independent experiments.



**Fig. 3.** NO scavenger (Carboxy-PTIO) decreased RSV induced VEGF production in HBEpC. HBEpC were infected with RSV, and VEGF in culture supernatants were estimated as described in methods. The data represent mean  $\pm$  SE of three independent experiments. Inclusion of NO scavenger Carboxy PTIO decreased VEGF levels to those of the control. \* $p < 0.001$  compared to control.



**Fig. 4.** Real-time electrical resistance across RSV (MOI of 0.005) infected and uninfected (control) primary bronchial epithelial monolayers. Carboxy PTIO (NO scavenger) was added to the cells at a concentration of 100  $\mu$ M. The addition of Carboxy PTIO recovered the decrease in resistance to the extent of control levels. The data represent mean  $\pm$  SD of three independent experiments and each condition was run in duplicate. \* $p < 0.05$  compared to control. <sup>§</sup> $p < 0.01$  compared to the control.

the airway lumen. This causes airway wall thickening and contributes to the formation of viscous mucus and airway plugging (6, 21). Thus, it is possible that increased airway permeability alterations contribute to the obstruction and the structural changes seen in the airways of infants who have severe RSV infection. The respiratory epithelium is the target for RSV infection and replication. RSV induced production of VEGF, and consequent changes in permeability play an important role in RSV-propagated inflammation and edema formation. We demonstrate that RSV infection causes permeability changes in the epithelial monolayer via a signaling pathway that involves the activation of HIF-1 $\alpha$  via NO release.

NO release in RSV infection has been demonstrated *in vitro* through the induction of iNOS after RSV infection (18). Increased NO production was abolished with UV-irradiated RSV or treatment with Ribavirin, suggesting that viral replication was necessary for the increased production of NO by infected epithelial cells (18). Viral infection induces type 1 interferon release. It is possible that the RSV stock we used in this study may have HeP-2 cell derived type 1 interferons. Type 1 interferons inhibit NO production in peritoneal macrophages and retinal epithelia cells (22, 23). In the study the RSV induced NO response significantly increased after 48 h of post infection. Therefore, we believe the NO response noticed was not interferon mediated. There has been evidence that NO can lead to the stabilization and induction of HIF-1 $\alpha$  in endothelial cells and other cell lines but not in epithelial cells (14, 24, 25). NO has been reported to induce HIF-1 $\alpha$ , HIF-1 DNA binding activity and gene expression under normoxic conditions via mechanisms that differ from those in hypoxia (24, 26, 27). HIF-1 $\alpha$  also mediates the expression of VEGF (28). To date several HIF-1 $\alpha$ target genes have been identified. They are involved in erythropoiesis and iron metabolism, glucose/energy metabolism, cell proliferation/viability decisions, vascular development/remodeling and vasomotor tone and others (29, 30). HIF-1 $\alpha$  controls several key aspects of inflammation including the swelling of injured tissue, and the ability of leukocytes to enter these sites (16). HIF-1 $\alpha$ target genes contribute to either protective or pathologic responses in several major disease states such as ischemic cardiovascular disorders, pulmonary hypertension and cancer (29, 30). In general, the stimulatory action of NO on accumulation of HIF-1 $\alpha$  implies a functional role of HIF-1 $\alpha$ during inflammatory settings (31).

VEGF-A is capable by itself of inducing the formation of tumor like blood vessels and connective tissue stroma. Further, VEGF-A is expressed not only in ma-

lignant tumors but also in healing wounds, in cellular immunity, and in chronic inflammatory diseases such as psoriasis and rheumatoid arthritis (32, 33). VEGF-A<sub>165</sub> is a multifunctional cytokine with a vascular permeabilizing activity some 50,000 times more potent than histamine (33). As far as is known, all the activities of VEGF-A are mediated through two receptor tyrosine kinases, VEGFR-1 and VEGFR-2, and through a nontyrosine kinase receptor, neuropilin (34, 35). The initial response to VEGF-A in all tissues studied consists of vascular hyperpermeability, fibrin deposition, and edema (36). Vascular hyperpermeability cause the leakage of plasma, and clotting of extravasated fibrinogen to form a fibrin gel that caused edema by trapping extravasated plasma, and serves as a provisional stroma for blood vessel and fibroblast migration (33, 37). VEGF antagonism reduces edema formation and damage after ischemia-reperfusion injury (38). It appears that VEGF production in RSV is responsible for the edema formation through changes in the epithelial monolayer itself and the adjacent vascular tissue. It could also be responsible for some of the structural changes observed after severe RSV infection. VEGF production was decreased by >90% when the RSV stock was exposed to UV light (10). The same investigators also demonstrated that rhinovirus, another respiratory virus, was unable to stimulate VEGF production indicating the VEGF production is at least in part specific to RSV infection.

This is the first study showing that HIF-1 $\alpha$  is stabilized during nonhypoxic conditions as a result of RSV infection. Whether the stabilization of HIF-1 $\alpha$  is specific to RSV or other bronchiolitis causing viruses needs further investigation.

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