Nicotinamide Exacerbates Hypoxemia in Ventilator-Induced Lung Injury Independent of Neutrophil Infiltration

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Abstract

Background
Ventilator-induced lung injury is a form of acute lung injury that develops in critically ill patients on mechanical ventilation and has a high degree of mortality. Nicotinamide phosphoribosyltransferase is an enzyme that is highly upregulated in ventilator-induced lung injury and exacerbates the injury when given exogenously. Nicotinamide (vitamin B3) directly inhibits downstream pathways activated by Nicotinamide phosphoribosyltransferase and is protective in other models of acute lung injury.

Methods
We administered nicotinamide i.p. to mice undergoing mechanical ventilation with high tidal volumes to study the effects of nicotinamide on ventilator-induced lung injury. Measures of injury included oxygen saturations and bronchoalveolar lavage neutrophil counts, protein, and cytokine levels. We also measured expression of nicotinamide phosphoribosyltransferase, and its downstream effectors Sirt1 and Cebpa, Cebpb, Cebpe. We assessed the effect of nicotinamide on the production of nitric oxide during ventilator-induced lung injury. We also studied the effects of ventilator-induced lung injury in mice deficient in C/EBPε.

Results
Nicotinamide treatment significantly inhibited neutrophil infiltration into the lungs during ventilator-induced lung injury, but did not affect protein leakage or cytokine production. Surprisingly, mice treated with nicotinamide developed significantly worse hypoxemia during mechanical ventilation. This effect was not linked to increases in nitric oxide production or alterations in expression of Nicotinamide phosphoribosyl transferase, Sirt1, or Cebpa and
Cebpb. Cebpe mRNA levels were decreased with either nicotinamide treatment or mechanical ventilation, but mice lacking C/EBPε developed the same degree of hypoxemia and ventilator-induced lung injury as wild-type mice.

Conclusions
Nicotinamide treatment during VILI inhibits neutrophil infiltration of the lungs consistent with a strong anti-inflammatory effect, but paradoxically also leads to the development of significant hypoxemia. These findings suggest that pulmonary neutrophilia is not linked to hypoxemia in ventilator-induced lung injury, and that nicotinamide exacerbates hypoxemia during VILI.

Introduction
Acute respiratory distress syndrome (ARDS) is a clinical syndrome characterized by an acute onset, bilateral infiltrates on chest x-ray, and profound hypoxemia [1]. Even with best supportive care, the mortality rate for this disease is about 25% [2]. Many patients who develop ARDS require mechanical ventilation, and unfortunately this intervention can precipitate or worsen disease progression [3]. The pressures and volumes used during positive-pressure mechanical ventilation of patients in the ICU can cause an acute lung injury termed ventilator-induced lung injury (VILI). Because of its direct clinical impact, VILI is an area of intense research [3–12].

One particularly active aspect of current research into the mechanisms of VILI focuses on the role of the enzyme nicotinamide phosphoribosyltransferase (NAMPT; also known as Pre-B cell Enhancing Factor). NAMPT is markedly upregulated in VILI and experimental studies indicate that it has diverse roles in augmenting acute lung injury by promoting alveolar permeability [13, 14], promoting neutrophil influx [7], inhibiting neutrophil apoptosis [15], and increasing oxidative stress [16]. Furthermore, intratracheal administration of recombinant NAMPT augments acute lung injury, whereas mice given FK866, a specific noncompetitive inhibitor of NAMPT, or lacking one allele of Nampt are protected from VILI [7, 17, 18]. Therefore, NAMPT is upregulated by VILI and aggravates acute lung injury, and its inhibition improves outcomes. The mechanisms by which NAMPT exerts these detrimental effects in VILI are unclear and understanding of the downstream pathways affected by NAMPT will be important for understanding the pathogenesis of acute lung injury.

NAMPT is a pleiotrophic enzyme with several different activities [19], but it is best characterized as the rate-limiting enzyme in the pathway that generates nicotinamide adenine dinucleotide (NAD+) [20]. NAD+ is a coenzyme for four families of enzymes: ADP-ribose cyclases, mono-ADP-ribosyltransferases, poly-ADP ribosyltransferases (PARPs) and the sirtuins, or type III histone deacetylases [21, 22], and some of these enzymes have been implicated in acute lung injury models. [23–30]. Nicotinamide (NAM), also known as vitamin B3, plays a dual role in the NAMPT/NAD+ pathway: NAM is the substrate for NAMPT and therefore the precursor of NAD+, but NAM also directly inhibits all NAD+-dependent enzymes by competing for the NAD+ binding site on these molecules [31]. Therefore, if NAMPT’s pro-inflammatory and injurious effects in VILI are related to generation of NAD+, one might predict that NAM administration could either exacerbate the proinflammatory effects of NAMPT by increasing production of NAD+, or ameliorate NAMPT’s effects by inhibiting downstream NAD+-dependent enzymes. Experimental data has suggested that it is the latter that occurs in vivo,
because NAM administration is anti-inflammatory in multiple models of inflammation [32–35] including models of acute lung injury. For example, niacin (the oral form of NAM) attenuated pro-inflammatory cytokine production in the lung in both bleomycin-induced [36] and sepsis-induced [37] acute lung injury, and improved survival in the sepsis model. NAM also decreased lung edema and damage in both ischemia/reperfusion [38] and LPS-induced [39, 40] acute lung injury models. We hypothesized that NAM would confer similar beneficial and anti-inflammatory effects in VILI. We found that NAM administration inhibited neutrophil infiltration into the lungs in our mouse model of VILI, consistent with a strong anti-inflammatory effect. To our surprise, however, NAM administration significantly worsened oxygenation. These findings suggest that: 1) neutrophil infiltration of the lungs is disassociated from the development of hypoxemia during VILI; and 2) NAM exacerbates hypoxemia in VILI through an as-yet undefined mechanism.

Materials and Methods

Mice

*Cebpe*−/− mice were provided by Dr. H. Phillip Koeffler (Cedars-Sinai Medical Center, Los Angeles, CA). Eight to 10 week old, sex matched 129/SvEv mice and male C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, ME).

Intubation, Mechanical Ventilation, and NAM administration

Mice to be mechanically ventilated were anesthetized with intraperitoneal injections of a mix of ketamine (Vedco Inc., Saint Joseph, MO) and dexmedetomidine (Pfizer, Irvine, CA) (75 mg/kg and 0.5 mg/kg respectively). Mice were orotracheally intubated and ventilated using an Inspira volume-controlled small animal ventilator (Harvard Apparatus, Holliston, MA) with a tidal volume of 20 ml/kg and a respiratory rate of 70 breaths/min with zero positive end-expiratory pressure. NAM (Sigma Aldrich, St. Louis, MO) or an equivalent volume of PBS were administered i.p. after 1 h of mechanical ventilation (MV), and MV was continued for a total of 6 h. PBS (500 μl, s.c.) was administered to all mice undergoing MV at 4 h of MV. Ketamine (50 mg/kg) was administered s.c. as needed (usually every 2–3 h). Mice were kept warm on a heating pad (38°C) (Hallowell EMC, Pittsfield, MA). Non-ventilated control mice received i.p. PBS or NAM at the same time as ventilated mice received PBS or NAM, and remained in their cages until the end of MV.

Pulse Oximetry

Arterial oxygen saturations were measured in MV mice using a MouseOX pulse oximeter (STARR Life Sciences, Oakmont, PA).

Serum and Bronchoalveolar Lavage (BAL)

After euthanasia with isoflurane overdose, the abdomen was opened, and peripheral blood was collected in heparinized syringes from the inferior vena cava and centrifuged to obtain serum. The trachea was exposed and cannulated with a 22 G IV cannula. 0.5 ml of PBS with 2 mM EDTA was instilled and aspirated two times. Cells were separated from supernatant, and the total number of cells was determined using a hemocytometer. Slides were then prepared from cell suspensions and stained with Diff-Quick (Fisher Scientific, Waltham, MA). A differential count was performed on 150 cells per animal, and expressed as an absolute number or percentage of total cells recovered. Serum and BAL supernatants were stored at -80°C for use in ELISAs and for total protein measurements. Lungs were perfused using 2 ml of PBS via
injection into the right ventricle, and then were removed. The left lung was separated, placed in RNAlater buffer, and stored at 4°C for RNA extraction. The right lung was removed, flash-frozen in liquid nitrogen, and stored at -80°C for further analysis.

**Detection of cytokines, total protein, and lung MPO**

The cytokine concentrations in BAL were determined using Mouse IL-1β ELISA (eBioscience, San Diego, CA), and Mouse keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP2) ELISA kits (R&D Systems, Minneapolis, MN). Total protein in serum and BAL samples was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). Myeloperoxidase concentrations were measured in lung homogenates using an MPO ELISA assay [41]. Two to three wells were used per sample for all assays.

**Quantitative RT-PCR**

The left lung was separated and placed in RNAlater buffer (Life Technologies, Grand Island, NY) and stored at 4°C. RNA was extracted with RNeasy Lipid Tissue Mini Kit (QIAGEN, Valencia, CA) and treated with deoxyribonuclease (QIAGEN, Valencia, CA) to eliminate genomic DNA. Two micrograms of purified total RNA was treated with gDNA elimination buffer and then reverse-transcribed into first-strand cDNA using oligo (deoxythymidine) primers, with QuantiTect reverse transcriptase (QIAGEN, Valencia, CA). Primers used for quantitative RT-PCR are murine *Sirt1* (Mm00490758), *Nos2* (Mm00440502), *Nampt* (Mm00451938), *Cebpa* (Mm00514283), *Cebpb* (Mm00843434), *Cebpe* (Mm02030363), and *Tubulin 1B* (Mm02030931) designed and purchased from Applied Biosystems. A 40-cycle PCR was carried out at 60°C annealing temperature in a MicroAmp Optical 96-well plate in BioRad iQ5 real-time PCR detection system. Amplicons and Taqman murine tubulin control expression assay used as an endogenous reference were detected using the relevant probes tagged with MGB quencher and FAM (carboxyfluorescein) dye (Life Technologies, Grand Island, NY)). Total of 100ng of RNA was loaded into each well. Samples were analyzed in duplicate.

**Histology and Immunostaining**

Lungs were perfused-fixed with PBS-buffered formalin through the trachea under 20 cm pressure for 5 min, incubated in fixative at room temperature for 48 hr, and embedded in paraffin. Immunohistochemical detection of rat anti-mouse Ly-6B.2 monoclonal antibody (7/4) (Abd Serotec, Raleigh, NC) was performed on 4-μm tissue sections. Staining was done manually using heat-induced epitope retrieval method in low Citrate 6.0 pH buffer. The staining was performed overnight in 4°C refrigeration at a 1/100 dilution for Ly-6B.2. The following day an anti-rat IgG, HRP linked secondary antibody (Cell Signaling Technology #7077, Beverly, MA) was applied at a dilution of 1/500 for 1 hour at lab room temperature. Tyramide signal amplification was performed using a TSA kit (Life Technologies, Grand Island, NY), and a streptavidin HRP conjugated antibody (Invitrogen) was incubated for 1 hour at lab room temperature at a 1/500 dilution. The staining was visualized using a DAB kit (Vector Labs, Burlingame, CA). Slides were subsequently counterstained with Mayer’s hematoxylin, dehydrated, cleared, and covered. Controls sections were processed with preimmune serum.

**Peripheral Complete Blood Counts and Differentials**

Whole blood was collected from the inferior vena cava of euthanized mice using syringes containing EDTA. The blood was immediately analyzed using a Hemavet 950 machine (Drew Scientific Inc.) for complete blood counts and automated differential analysis of white blood cells.
Detection of C/EBPε protein

Mice were treated with mechanical ventilation and PBS/nicotinamide i.p. as described in Methods (PBS, NAM), and control mice received only PBS or nicotinamide i.p (cPBS, cNAM). All animals were euthanized at the end of the experiment. Lungs were perfused via the right ventricle, and then removed en bloc from the thorax and frozen in liquid nitrogen. Later, frozen lungs were thawed on ice and homogenized in 1 ml of lysis buffer (50 mM HEPES, 100 mM NaCl. 1mM EDTA, 1mM Na3VO4, 10% Glycerol, 0.5% NP-40, protease Inhibitor mix 1%) 10 µg of total lung lysate were loaded per lane for SDS-PAGE, and proteins were transferred to nitrocellulose and probed for C/EBPε using primary antibody against C/EBPε (Santa Cruz Biotechnology Inc.) and corresponding secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch). The blots were visualized using SuperSignal West Pick Chemiluminescent substrate (Thermo Scientific).

Statistical Analysis

All data were analyzed with the Prism 4.03 statistical program. To compare differences in BAL cell numbers, cytokine levels, and BAL/serum protein ratios, the 2-tailed Student t test (at 95% confidence interval) was used to compare unpaired samples between experimental groups. For experiments involving 3 or more groups, we used 1-way ANOVA with the Tukey post hoc test. For experiments comparing oxygen saturations between groups at different time points, we used the 2-way ANOVA with the Bonferroni post hoc test. A value of p < 0.05 was considered statistically significant.

Ethics Statement

The study was approved by and all animal experiments were conducted according to the Cedars-Sinai Medical Center Institutional Animal Care and Use Committee guidelines.

Results

Nicotinamide decreases BAL polymorphonucleocytes (PMNs) but causes significant hypoxemia in ventilator-induced lung injury (VILI)

We hypothesized that systemic nicotinamide (NAM) would exert anti-inflammatory properties and therefore be protective in a mouse model of ventilator-induced lung injury. Indeed, although NAM did not affect the mechanical ventilation (MV)-mediated increase in BAL protein (Fig 1A), indicating it did not impact the mechanical injury, NAM significantly decreased neutrophil influx in the BAL and lung tissue (Fig 1B and 1C and Fig 2). These findings indicate that alveolar edema in this model was not dependent on alveolar injury due to neutrophil infiltration. BAL concentrations of chemokines KC and MIP2 were not affected by NAM treatment (Fig 1D and 1E], suggesting that NAM’s inhibitory effect on neutrophil infiltration of the lung in VILI was not due to inhibition of chemokine expression. Consistent with earlier findings [42], peripheral blood PMN counts were unchanged by NAM treatment (S1 Fig) suggesting that an overall decrease in PMN counts in the bloodstream is not the cause of decreased PMN infiltration into the lungs.

Surprisingly, in comparison to PBS-treated mice, NAM-treated mice developed significant, progressive hypoxemia while on mechanical ventilation (Fig 1F). Although reduced neutrophil influx was consistent with our proposed anti-inflammatory effect of NAM, fewer neutrophil likely do not account for the increased hypoxemia with NAM+MV. In LPS+MV-induced ALI, IL-1β caused hypoxemia [43], therefore we measured IL-1β in BAL. However, we did not observed any increase of IL-1β (Fig 1G), suggesting that IL-1β is not responsible for the
Fig 1. Nicotinamide decreases BAL PMNs but causes significant hypoxemia in VILI. (A) Mice were anesthetized and placed on mechanical ventilation (MV) with tidal volumes of 20 ml/kg and zero PEEP. After
progressive hypoxemia induced by NAM+MV. In addition, BAL TNF-α and IL-6 were increased with mechanical ventilation, and not affected by NAM (S2 Fig). The responses of neutrophil numbers and oxygen saturations to NAM treatment displayed a dose-response effect, whereas edema did not (Fig 3A–3C). Because the effect is consistent and dose-dependent, the higher NAM dose of 400 mg/kg was used for all subsequent experiments.

Nicotinamide decreases lung expression of C/EPBε in VILI

NAM, NAMPT, and NAD+ are components of an integrated signaling pathway. We assessed if NAM affected the expression of Nampt, the histone deacetylase SIRT1 (which requires NAD + as a cofactor, and is directly inhibited by NAM), and SIRT1-regulated transcription factors Cebpa, Cebpb, and Cebpe. MV mediated a significant increase in the expression of Nampt, as seen by others [7, 44], and Cebpb, but did not influence the levels of Sirt1 or Cebpa mRNA expression (Fig 3). NAM did not affect the basal and MV-regulated levels of these four transcripts. In contrast, expression of Cebpe which functions in neutrophil development and function [45], was significantly decreased by either NAM treatment or MV, and the combination of NAM and MV resulted in about a 10-fold reduction in Cebpe mRNA levels compared with controls (Fig 4E). C/EBPε protein concentrations in lung tissue (S3 Fig) were variable, and there was no consistent effect of NAM or MV on C/EBPε protein levels.

C/EBPε-deficient mice have decreased lung PMNs but equivalent hypoxemia in VILI

Because both NAM treatment and MV downregulated Cebpe mRNA levels in lung tissue, we assessed if decreased C/EBPε was linked to hypoxemia or inhibition of lung neutrophilia in VILI. Wildtype (WT) and Cebpe−/− mice were placed on mechanical ventilation for 6 h, and oxygen saturations were measured every hour. Cebpe−/− mice demonstrated lower oxygen saturations at earlier times (180 and 240 min of MV), but the differences in hypoxemia were not seen at later time points (Fig 5A). These findings suggest that C/EBPε deficiency was not responsible for the hypoxemia observed in mice with NAM treatment on MV. PMN infiltration into the lung with VILI was highly suppressed in Cebpe−/− mice (Fig 5C), consistent with established Cebpe−/− phenotypes [46]. BAL protein levels were increased equally in WT and Cebpe−/− mice with MV, again demonstrating a disconnect between PMN infiltration and alveolar leakage in VILI (see Figs 1 and 2). BAL IL-1, MIP2, and KC concentrations were similar between WT and Cebpe−/− mice after MV (data not shown).

Nicotinamide decreases lung expression of NOS2 in VILI

Because nitric oxide levels are increased in the BAL fluid of patients with acute lung injury [47] and because NAM has been reported to inhibit nitric oxide synthase 2 (NOS2) expression [48], we examined the effect of NAM on Nos2 mRNA levels. Nos2 expression was increased with
Fig 2. Nicotinamide inhibits neutrophil infiltration of lungs during VILI. Mice were anesthetized and placed on mechanical ventilation (MV) and received i.p. nicotinamide or PBS as described in Fig 1. All mice were euthanized at the end of 6 hours mechanical ventilation or spontaneous breathing (control mice),
which was 5 hours after NAM or PBS administration, and lungs were harvested, fixed, and embedded. Sections of lungs were incubated with GR-1, an antibody for murine neutrophils. Stained neutrophils (white arrows) are white in these reverse images of mice treated with: (A) PBS only; (B) Nicotinamide only; (C) PBS and mechanical ventilation; and (D) Nicotinamide and mechanical ventilation. Images are shown at 10x (left panels) and 40x (right panels) magnifications.

Fig 3. Effects of nicotinamide on hypoxemia and PMNs are dose-dependent. Mice were anesthetized and placed on mechanical ventilation as described in Fig 1. After one hour of mechanical ventilation, mice were injected intraperitoneally with PBS or NAM at doses of 50 mg/kg (NAM50+MV), 200 mg/kg (NAM200+MV) or 400 mg/kg (NAM400+MV). (A) Oxygen saturation was measured each hour. Significance is indicated for comparisons between NAM400 and NAM50 (** p < 0.01, *** p < 0.001) and between NAM400 and NAM200 (## p < 0.05, ### p < 0.001). Mice were euthanized at the end of 6 hours mechanical ventilation, and bronchoalveolar lavage (BAL) fluid was assayed for: (B) protein concentration and (C) neutrophil (PMNs) numbers and percentages of total BAL cells. PBS and NAM400 data are repeated from Fig 1 for comparison with NAM50 and NAM200. Data are representative of two separate experiments for NAM50 and NAM200, and four separate experiments for PBS and NAM400. Significance is indicated for comparisons between PBS and PBS+MV, and between PBS+MV and all NAM+MV doses. * p < 0.05, ** p < 0.01, *** p < 0.001.

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Fig 4. Nicotinamide and mechanical ventilation decrease lung expression of Cebpe. Mice were anesthetized and placed on mechanical ventilation as described in Fig 1 and received either PBS (PBS+MV) or nicotinamide at 400 mg/kg (NAM+MV) after 1 hour of mechanical ventilation. Control mice were allowed to continue spontaneously breathing and received PBS or nicotinamide at the same time as mice on mechanical ventilation (PBS, NAM). Mice were euthanized at the end of 6 hours mechanical ventilation, and the left lung was placed in RNeasy buffer and homogenized. RT-PCR was performed using TaqMan primers for (A) Nampt; (B) Sirt1; (C) Cebpa; (D) Cebpb; and (E) Cebpe. Significance is indicated for comparisons between PBS and PBS+MV, and between PBS+MV and NAM+MV unless otherwise shown. * p < 0.05, ** p < 0.01, *** p < 0.001.

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MV, and significantly decreased by NAM treatment (Fig 6A). We measured BAL nitrite concentrations and found that MV increased BAL nitrites, as reported [47, 49], but that NAM had no effect on MV-induced increases in BAL nitrites (Fig 6B). Therefore, we conclude that the effects of NAM on hypoxemia in our VILI model cannot be attributed to specific effects on lung NOS2 activity.

Discussion

The main findings of this research are that nicotinamide treatment during VILI inhibits neutrophil infiltration of the lungs but, paradoxically, also leads to the development of significant hypoxemia. Both PMN inhibition and hypoxemia in response to NAM treatment during VILI occur in a dose-dependent fashion. The observation that PMN infiltration can be distinct from the development of hypoxemia in VILI challenges the assumption that individual measures of acute lung injury can serve as surrogates for the disease process as a whole, and suggests that the mechanisms behind hypoxemia in acute lung injury may be more complicated than previously thought.

We were interested in exploring the effects of NAM in VILI as an inhibitor of NAMPT-related pathways because of the growing body of evidence related to the role of NAMPT in this disease. NAMPT exerts a pro-inflammatory effect in VILI, increasing neutrophils, edema, BAL pro-inflammatory cytokines, and histological damage [7, 14, 50], and anti-NAMPT therapy with specific inhibitors improves these measures [18]. We hypothesized that NAM would be protective in VILI because it is a direct inhibitor of key enzymes that are downstream of and controlled by NAMPT [19–21, 31] and because it has been shown to be strongly anti-inflammatory in vitro [32, 33, 48] and in vivo in models of inflammatory disease [34], including other models of acute lung injury [35, 40, 51–54]. Indeed, we found a significant and dose-dependent inhibitory effect of NAM treatment during VILI on BAL PMN concentrations. Lung MPO concentrations were measured as a reflection of total lung PMNs, and were also reduced, and this was confirmed on histological examination, ruling out the possibility that NAM treatment merely prevented PMNs from reaching the alveolar space but did not inhibit PMNs from entering the lung interstitium during VILI. BAL cytokines were not affected by NAM treatment, suggesting that NAM’s effect is related to the inhibition of neutrophil transmigration out of the vasculature rather than an inhibition of chemokine production in the lungs.

Our results are in contrast to other work on NAM’s effects on neutrophils. Skokawa et al. showed that NAM administration induced neutrophilic granulopoiesis in vitro through high intracellular NAMPT and NAD+ levels and subsequent induction of SIRT1 and C/EBP transcription factor activation [20]. Additionally, researchers from our group demonstrated that NAM treatment in a S. aureus skin infection model strongly enhanced the ability of neutrophils to clear infection due to increased bactericidal activity with NAM treatment, which was dependent on an increased expression of C/EBPε in neutrophils [42]. However, we suggest that NAM’s inhibitory effects on neutrophil transmigration to an area of sterile injury as is seen in...
the lungs in VILI may be distinct from NAM's effects on neutrophil maturation and/or neutrophil activity against bacterial infection, and that this differential effect may be an important area for further research.

The finding that NAM inhibited PMN infiltration into the lung during VILI, but at the same time caused progressive and dose-dependent decreases in oxygen saturations, was entirely unexpected. Many models of lung injury measure PMN infiltration, alveolar edema, and
inflammatory cytokines as indicators of the degree of lung injury, and improvements in these parameters are taken as evidence of improvement in acute lung injury [55–57]. Oxygen saturation is rarely reported as a measure of an acute lung injury model in mice, although hypoxemia is one of the most important clinical manifestations of acute lung injury. In this paper we found that PMN infiltration, a hallmark of acute lung inflammation and injury, is completely disassociated from hypoxemia. This is difficult to reconcile with prior research that has established a clear role for neutrophils in the development of acute lung injury. Neutrophil depletion has been shown to decrease alveolar edema and inflammatory cytokine concentrations in animal models [58–60], and mechanisms of neutrophil-induced lung injury are well established [61–66], and neutrophil infiltration of the lungs and protein-rich alveolar edema are hallmarks of the development of acute lung injury, both in humans and in animal models [55–57].

However, we recently reported a similar finding in a “two-hit” lipopolysaccharide and MV model of acute lung injury, in which abrogation of IL-1β signaling prevented the development of hypoxemia but did not affect the level of PMN infiltration into the lung or the development of alveolar edema [43]. In that study, we also depleted PMNs from mice using anti-neutrophil antibodies, and found that the development of hypoxemia occurred at the same rate as control mice. It is unclear why hypoxemia is distinct from other measures of acute lung injury; because oxygen saturations are rarely measured in small animals studies of acute lung injury, little is known about this variable in relation to other measures of acute lung injury. One possible explanation for this disconnect could be an alteration in patterns of pulmonary blood flow that leads to increased ventilation-perfusion mismatching. NOS2 is a determinant of pulmonary vascular blood flow [67–69], and nitric oxide production is increased in acute lung injury [47]; we therefore explored whether exogenous NAM could affect Nos2 expression levels in the lung. Indeed, we found that MV increased Nos2 expression in the lung, and that NAM administration strongly inhibited this increase. However, although BAL nitrite levels (as a marker of lung NO production) were increased with MV, as has been described in other work [47], NAM treatment during MV did not decrease BAL nitrite levels. It is possible that compartmentalized effects of NAM on Nos2 transcription may be more important than global effects in the lung that are demonstrated in BAL; for example, if endothelial NOS2 production is important for maintaining ventilation-perfusion matching in VILI, this may not be reflected in BAL nitrites. At this point, the mechanism for NAM-associated hypoxemia in VILI remains unclear. We are actively exploring this question because of the obvious clinical relevance, and because prior work suggests that the NAM/NAMPT/NAD axis is important in VILI [7, 13, 14, 16, 44, 50], and this line of inquiry may help to explain why.

We investigated the impact of MV and NAM treatment on expression of molecules in this axis: the enzyme NAMPT; the histone deacetylase Sirt1 (which is activated by NAMPT and inhibited by NAM); and C/EBPα, β, and ε, which are three transcription factors modulated by Sirt1. We found a marked decrease in Cebpe mRNA expression with either MV or NAM treatment, and the effect was additive (Fig 4). To explore this further, we tested the effects of MV in C/EBPε-deficient mice, which have markedly dysfunctional neutrophils due to a lack of C/EBPε. However, although lung neutrophilia was absent in Cebpe−/− mice (Fig 5), hypoxemia developed over time to the same extent in both WT and Cebpe−/− mice on MV. This suggests that inhibition of C/EBPε with NAM treatment was not the cause of progressive hypoxemia in NAM-treated mice. Further investigation will be needed to understand these complex relationships, but the lack of association between PMN infiltration into the lungs and the development of hypoxemia again suggests that these two components of acute lung injury may not be connected.

A caveat to our work is that the relationships between intracellular and extracellular NAMPT and NAD+ are poorly understood. Extracellular NAMPT has been shown to be a
robust producer of systemic NAD+ [70], although its physiological role is not clear. Exogenous NAM administration increases intracellular NAD+ concentrations in vitro and in human volunteers, suggesting that exogenous NAM can affect the intracellular balance of NAD+ [20, 21]. We did not measure intracellular or extracellular NAD+ concentrations, and so the effects of NAM administration on this parameter in our model of VILI are unknown. It is possible that one concentration of exogenous NAM provides more substrate to drive the formation of NAD+, whereas another concentration of NAM exerts predominantly inhibitory effects on downstream NAD+ dependent enzymes. However, our data demonstrated a consistent dose-dependent effect with increasing NAM doses. The complex relationships between intra- and extracellular NAMPT, NAD+, and NAM in VILI and other diseases will require further work.

In conclusion, we explored the anti-inflammatory effects of nicotinamide administration in a mouse model of VILI and found that although PMN infiltration of the lungs was significantly inhibited by NAM treatment, severe hypoxemia also developed in these mice. This finding is novel and suggests a new area of investigation, particularly in relation to the NAM/NAMPT/NAD+ axis in acute lung injury and the mechanisms of hypoxemia in this disease process. Further research will be directed at determining the role of NAMPT and its downstream effectors specifically in the development of hypoxemia in VILI.

Supporting Information

S1 Fig. Nicotinamide does not affect neutrophil counts in peripheral blood. Mice were anesthetized and placed on mechanical ventilation (MV) with tidal volumes of 20 ml/kg and zero PEEP. After 1 hour of mechanical ventilation, mice received intraperitoneal injections of either 400 mg/kg nicotinamide (NAM+MV) or an equivalent volume of PBS (PBS+MV), and mechanical ventilation was continued for a total of 6 hours; control mice received intraperitoneal NAM or PBS at the same as the mice on mechanical ventilation (PBS, NAM) but were not anesthetized. All mice were euthanized at the end of 6 hours mechanical ventilation or spontaneous breathing (control mice). Whole blood was collected in syringes containing EDTA from the inferior vena cava, and analyzed for complete blood including leukocyte counts and types of leukocytes (automated differential). Differences in neutrophil counts among groups did not reach statistical significance. n = 6-7/group.

S2 Fig. Nicotinamide does not affect BAL IL-6 or TNF alpha concentrations during VILI. Mice were anesthetized and placed on mechanical ventilation (MV) with tidal volumes of 20 ml/kg and zero PEEP. After 1 hour of mechanical ventilation, mice received intraperitoneal injections of either 400 mg/kg nicotinamide (NAM+MV) or an equivalent volume of PBS (PBS+MV), and mechanical ventilation was continued for a total of 6 hours; control mice received intraperitoneal NAM or PBS at the same as the mice on mechanical ventilation (PBS, NAM) but were not anesthetized. All mice were euthanized at the end of 6 hours mechanical ventilation or spontaneous breathing (control mice), and bronchoalveolar lavage (BAL) fluid was assayed for: (A) IL-6 and (B) TNF. Differences in IL-6 and TNF concentrations between PBS +MV and NAM+MV groups did not reach statistical significance. N.D. signifies groups for which IL-6 and TNF concentrations were below the limit of detection. n = 6-7/group.

S3 Fig. Nicotinamide does not affect lung C/EBP epsilon concentrations. Mice were anesthetized and placed on mechanical ventilation (MV) with tidal volumes of 20 ml/kg and zero PEEP. After 1 hour of mechanical ventilation, mice received intraperitoneal injections of either 400 mg/kg nicotinamide (NAM) or an equivalent volume of PBS (PBS), and mechanical
ventilation was continued for a total of 6 hours; control mice received intraperitoneal NAM or PBS at the same as the mice on mechanical ventilation (cPBS, cNAM) but were not anesthetized. All mice were euthanized at the end of 6 hours mechanical ventilation or spontaneous breathing (control mice). Whole lungs were homogenized in lysis buffer and analyzed via Western for C/EBP epsilon protein.

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Author Contributions

Conceived and designed the experiments: HJ TC MA KS PK GL WP. Performed the experiments: HJ JY AB RK PK GL CT. Analyzed the data: HJ JY AB PK GL CT KS TC WP. Contributed reagents/materials/analysis tools: HJ MA GL. Wrote the paper: HJ JY TC KS MA PK GL AB WP.

References

1. Ranieri VM, Rubenfeld GD, Thompson BT, Ferguson ND, Caldwell E, Fan E, et al. Acute respiratory distress syndrome: the Berlin Definition. JAMA. 2012; 307(23):2526–33. doi: 10.1001/jama.2012.5669 PMID: 22797452
2. Spragg RG, Bernard GR, Checkley W, Curtis JR, Gajic O, Guyatt G, et al. Beyond mortality: future clinical research in acute lung injury. Am J Respir Crit Care Med. 2010; 181(10):1121–7. doi: 10.1164/rccm.201001-0024WS PMID: 20224063
3. Villar J, Blanco J, Zhang H, Slutsky AS. Ventilator-induced lung injury and sepsis: two sides of the same coin? Minerva Anestesiol. 2011; 77(6):647–53. PMID:21617628
4. Wurfel MM. Microarray-based analysis of ventilator-induced lung injury. Proc Am Thorac Soc. 2007; 4 (1):77–84. PMID: 17202295
5. Wösten-van Asperen RM, Lutter R, Specht PA, van Woensel JB, van der Loos CM, Florquin S, et al. Ventilator-induced inflammatory response in lipopolysaccharide-exposed rat lung is mediated by angiotensin-converting enzyme. Am J Pathol. 2010; 176(5):2219–27. doi:10.2353/ajpath.2010.090565 PMID: 20304959
6. Kuipers MT, Aslami H, Janczy JR, van der Sluijs KF, Vlaar AP, Wolthuis EK, et al. Ventilator-induced lung injury is mediated by the NLRP3 inflammasome. Anesthesiology. 2012; 116(5):1104–15. doi: 10.1097/ALN.0b013e318251bb4c1 PMID: 22531249
7. Hong SB, Huang Y, Moreno-Vinasco L, Sammani S, Moitra J, Barnard JW, et al. Essential role of pre-B-cell colony enhancing factor in ventilator-induced lung injury. Am J Respir Crit Care Med. 2008; 178 (6):805–17. doi: 10.1164/rccm.200712-1822OC PMID: 18658108
8. Frank JA, Wray CM, McAuley DF, Schwendener R, Matthay MA. Alveolar macrophages contribute to alveolar barrier dysfunction in ventilator-induced lung injury. Am J Physiol Lung Cell Mol Physiol. 2006; 291(6):L1191–8. PMID: 16877636
9. Eyal FG, Hamm CR, Parker JC. Reduction in alveolar macrophages attenuates acute ventilator-induced lung injury in rats. Intensive Care Med. 2007; 33(7):1212–8. PMID: 17468847
10. Dhanireddy S, Altermeier WA, Matute-Bello G, O’Mahony DS, Glenny RW, Martin TR, et al. Mechanical ventilation induces inflammation, lung injury, and extra-pulmonary organ dysfunction in experimental pneumonia. Lab Invest. 2006; 96(7):730–9. PMID: 16855596
11. Altermeier WA, Matute-Bello G, Frevert CW, Kawata Y, Kajikawa O, Martin TR, et al. Mechanical ventilation with moderate tidal volumes synergistically increases lung cytokine response to systemic endotoxin. Am J Physiol Lung Cell Mol Physiol. 2004; 287(3):L533–42. PMID: 15145786
12. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. The Acute Respiratory Distress Syndrome Network. N Engl J Med. 2000; 342(18):1301–8. PMID: 10793162
13. Ye SQ, Zhang LQ, Adyshev D, Usatyuk PV, Garcia AN, Lavoie TL, et al. Pre-B-cell-colony-enhancing factor is critically involved in thrombin-induced lung endothelial cell barrier dysregulation. Microvasc Res. 2005; 70(3):142–51. PMID: 16188281

14. Liu P, Li H, Cepeda J, Zhang LQ, Cui X, Garcia JG, et al. Critical role of PBEF expression in pulmonary cell inflammation and permeability. Cell Biol Int. 2009; 33(1):19–30. doi:10.1016/j.cellbi.2008.10.015 PMID: 18996492

15. Jia SH, Li Y, Parodo J, Kapus A, Fan L, Rotstein OD, et al. Pre-B cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. J Clin Invest. 2004; 113(9):1318–27. PMID: 15124023

16. Zhang LQ, Adyshev DM, Singleton P, Li H, Cepeda J, Huang SY, et al. Interactions between PBEF and oxidative stress proteins—a potential new mechanism underlying PBEF in the pathogenesis of acute lung injury. FEBS Lett. 2008; 582(13):1802–8. doi:10.1016/j.febslet.2008.04.061 PMID: 18486613

17. Matsuda A, Yang WL, Jacob A, Aziz M, Matsuo S, Matsutani T, et al. FK866, a Visfatin Inhibitor, Protects Against Acute Lung Injury After Intestinal Ischemia-Reperfusion in Mice via NF-κB Pathway. Ann Surg. 2013.

18. Moreno-Vinasco L, Quijada H, Sammani S, Siegler J, Letsiou E, Deaton R, et al. Nicotinamide phosphoribosyltransferase inhibitor is a novel therapeutic candidate in murine models of inflammatory lung injury. Am J Respir Cell Mol Biol. 2014.

19. Gallí M, Van Gool F, Rongvaux A, Andris F, Leo O. The nicotinamide phosphoribosyltransferase: a molecular link between metabolism, inflammation, and cancer. Cancer Res. 2010; 70(1):8–11. doi: 10.1158/0008-5472.CAN-09-2462 PMID: 20028851

20. Skokowa J, Lan D, Thakur BK, Wang F, Gupta K, Cario G, et al. NAMPT is essential for the G-CSF-induced myeloid differentiation via a NAD(+)-sirtuin-1-dependent pathway. Nat Med. 2009; 15(2):151–8. doi: 10.1038/nm.1913 PMID: 19182797

21. Van Gool F, Gallí M, Gueydan C, Kruys V, Prevot PP, Bedalov A, et al. Intracellular NAD levels regulate tumor necrosis factor protein synthesis in a sirtuin-dependent manner. Nat Med. 2009; 15(2):206–10. doi: 10.1038/nm.1906 PMID: 19151729

22. Legutko A, Lekeux P, Bureau F. NAD+-Consuming Enzymes in the Regulation of Lung Immune Responses. The Open Immunology Journal. 2009; 2:42–51.

23. Liaudet L, Pacher P, Mabley JG, Virág L, Mabley JG, et al. Activation of poly(ADP-Ribose) polymerase-1 is a central mechanism of lipopolysaccharide-induced acute lung inflammation. Am J Respir Crit Care Med. 2002; 166(3):372–7. PMID: 11818323

24. Ahmad SF, Zhaoir KM, Ansari MA, Korashy HM, Bakheet SA, Ashour AE, et al. The role of poly(ADP-ribose) polymerase-1 inhibitor in carrageenan-induced lung inflammation in mice. Mol Immunol. 2014.

25. Kapoor K, Singla E, Sahu B, Naura AS. PARP inhibitor, olaparib ameliorates acute lung and kidney injury upon intratracheal administration of LPS in mice. Mol Cell Biochem. 2014.

26. Li T, Zhang J, Feng J, Li Q, Wu L, Ye Q, et al. Resveratrol reduces acute lung injury in a LPS-induced sepsis mouse model via activation of Sirt1. Mol Med Rep. 2013; 7(6):1889–95. doi: 10.3892/mmr.2013.2609 PMID: 23625030

27. Wu CT, Yu HP, Chung CY, Lau YT, Liao SK. Attenuation of lung inflammation and pro-inflammatory cytokine production by resveratrol following trauma-hemorrhage. Chin J Physiol. 2008; 51(6):363–8. PMID: 19280880

28. Yeh DY, Fu YH, Yang YC, Wang JJ. Resveratrol alleviates lung ischemia and reperfusion-induced pulmonary capillary injury through modulating pulmonary mitochondrial metabolism. Transplant Proc. 2014; 46(4):1131–4. doi: 10.1016/j.transproceed.2013.11.094 PMID: 24815145

29. Zhang Z, Chen N, Liu JB, Wu JB, Zhang J, Zhang Y, et al. Protective effect of resveratrol against acute lung injury induced by lipopolysaccharide via inhibiting the myd88-dependent Toll-like receptor 4 signaling pathway. Mol Med Rep. 2014; 10(1):101–6. doi: 10.3892/mmr.2014.2226 PMID: 24818579

30. Zhang HX, Duan GL, Wang CN, Zhang YQ, Zhu XY, Liu YJ. Protective effect of resveratrol against endotoxin-induced lung injury involves the reduction of oxidative/nitrative stress. Pulm Pharmacol Ther. 2014; 27(2):150–5. doi: 10.1016/j.pupt.2013.07.007 PMID: 23921197

31. Belenky P, Bogan KL, Brenner C. NAD+ metabolism in health and disease. Trends Biochem Sci. 2007; 32(1):12–9. PMID: 17161604

32. Ungerstedt JS, Blömbäck M, Söderström T. Nicotinamide is a potent inhibitor of proinflammatory cytokines. Clin Exp Immunol. 2003; 131(1):48–52. PMID: 12513985

33. Ungerstedt JS, Heimersson K, Söderström T, Hansson M. Nicotinamide inhibits endotoxin-induced monocyte tissue factor expression. J Thromb Haemost. 2003; 1(12):2554–60. PMID: 14675092
34. Suzuki E, Okuda H, Nishida K, Fujimoto S, Nagasawa K. Protective effect of nicotinamide against poly(ADP-ribose) polymerase-1-mediated astrocyte death depends on its transporter-mediated uptake. Life Sci. 2010; 86(17–18):676–82. doi: 10.1016/j.lfs.2009.10.011 PMID: 19896489

35. Kwon WY, Suh GJ, Kim KS, Kwak YH. Niacin attenuates lung inflammation and improves survival during sepsis by downregulating the nuclear factor-κB pathway. Crit Care Med. 2011; 39(2):328–34. doi: 10.1097/CCM.0b013e3181feee4 PMID: 20975550

36. Gurujeyalakshmi G, Wang Y, Giri SN. Taurine and niacin block lung injury and fibrosis by down-regulating bleomycin-induced activation of transcription nuclear factor-kappaB in mice. J Pharmacol Exp Ther. 2000; 293(1):82–90. PMID: 10734156

37. Su CF, Liu DD, Kao SJ, Chen HI. Nicotinamide abrogates acute lung injury caused by ischaemia/reperfusion. Eur Respir J. 2007; 30(2):199–204. PMID: 17504797

38. Fernandes CA, Fievez L, Ucakar B, Neyrinck AM, Fillee C, Huaux F, et al. Nicotinamide enhances apoptosis of G(M)-CSF-treated neutrophils and attenuates endotoxin-induced airway inflammation in mice. Am J Physiol Lung Cell Mol Physiol. 2011; 300(3):L354–61. doi:10.1152/ajplung.00198.2010 PMID: 21131399

39. Gill SE, Huizar I, Bench EM, Sussman SW, Wang Y, Khokha R, et al. Tissue inhibitor of metalloproteinases 3 regulates resolution of inflammation following acute lung injury. Am J Pathol. 2010; 176(1):64–73. doi: 10.2353/ajpath.2010.090158 PMID: 20008147

40. Kyme P, Thoennissen NH, Tseng CW, Thoennissen GB, Wolf AJ, Shimada K, et al. C/EBPε mediates nicotinamide-enhanced clearance of Staphylococcus aureus in mice. J Clin Invest. 2012; 122(9):3316–29. doi: 10.1172/JCI62070 PMID: 22922257

41. Lekstrom-Himes J, Xanthopoulos KG. CCAAT/enhancer binding protein epsilon is critical for effective neutrophil-mediated response to inflammatory challenge. Blood. 1999; 93(9):3096–105. PMID: 10216107

42. Yamanaka R, Barlow C, Lekstrom-Himes J, Castilla LH, Liu PP, Eckhaus M, et al. Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice. Proc Natl Acad Sci U S A. 1997; 94(24):13187–92. PMID: 9371821

43. Pellat-Deceunynck C, Wietzerbin J, Drapier JC. Nicotinamide inhibits nitric oxide synthase mRNA induction in activated macrophages. Biochem J. 1994; 297 (Pt 1):53–8.

44. Kobayashi A, Hashimoto S, Kooguchi K, Kitamura Y, Onodera H, Urata Y, et al. Expression of inducible nitric oxide synthase and inflammatory cytokines in alveolar macrophages of ARDS following sepsis. Chest. 1998; 113(6):1632–9. PMID: 9631804

45. Ye SQ, Simon BA, Maloney JP, Zambelli-Weiner A, Gao L, Grant A, et al. Pre-B-cell colony-enhancing factor as a potential novel biomarker in acute lung injury. Am J Respir Crit Care Med. 2005; 171 (4):361–70. PMID: 15579727

46. Wang QJ, Giri SN, Hyde DM, Li C. Amelioration of bleomycin-induced pulmonary fibrosis in hamsters by combined treatment with taurine and niacin. Biochem Pharmacol. 1991; 42(5):1115–22. PMID: 1714734

47. Wang QJ, Giri SN, Hyde DM, Nakashima JM, Javadi I. Niacin attenuates bleomycin-induced lung fibrosis in the hamster. J Biochem Toxicol. 1990; 5(1):13–22. PMID: 16982227

48. Gurujeyalakshmi G, Wang Y, Giri SN. Taurine and niacin block lung injury and fibrosis by down-regulating bleomycin-induced activation of transcription nuclear factor-kappaB in mice. J Pharmacol Exp Ther. 2000; 293(1):82–90. PMID: 10734156
54. Fernandes CA, Fievez L, Ucakar B, Neyrinck AM, Filée C, Huaux F, et al. Nicotinamide enhances apoptosis of G(M)-CSF-treated neutrophils and attenuates endotoxin-induced airway inflammation in mice. Am J Physiol Lung Cell Mol Physiol. 2011; 300(3):L354–61. doi: 10.1152/ajplung.00198.2010 PMID: 21131999

55. Matute-Bello G, Downey G, Moore BB, Groshong SD, Matthay MA, Slutsky AS, et al. An official American Thoracic Society workshop report: features and measurements of experimental acute lung injury in animals. Am J Respir Cell Mol Biol. 2011; 44(5):725–38. doi: 10.1165/rcmb.2009-0210ST PMID: 21531958

56. Matthay MA, Ware LB, Zimmerman GA. The acute respiratory distress syndrome. J Clin Invest. 2012; 122(8):2731–40. doi: 10.1172/JCI60331 PMID: 22850883

57. Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2008; 295(3):L379–99. doi: 10.1152/ajplung.00010.2008 PMID: 18621912

58. Bhatia M, Saluja AK, Hofbauer B, Lee HS, Frossard JL, Steer ML. The effects of neutrophil degranulation on a completely noninvasive model of acute pancreatitis-associated lung injury. Int J Pancreatol. 1998; 24(2):77–83. PMID: 9816540

59. Soehnlein O, Oehmcke S, Ma X, Rothfuchs AG, Frithiof R, van Rooijen N, et al. Neutrophil degranulation mediates severe lung damage triggered by streptococcal M1 protein. Eur Respir J. 2008; 32(2):405–12. doi: 10.1183/09031936.00173207 PMID: 18321926

60. Abraham E, Carmody A, Shenkar R, Arcaroli J. Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2000; 279(6):L1137–45. PMID: 11076804

61. Lee WL, Downey GP. Neutrophil activation and acute lung injury. Curr Opin Crit Care. 2001; 7(1):1–7. PMID: 11373504

62. Grommes J, Soehnlein O. Contribution of neutrophils to acute lung injury. Mol Med. 2011; 17(3–4):293–307.

63. Zemans RL, Colgan SP, Downey GP. Transepithelial migration of neutrophils: mechanisms and implications for acute lung injury. Am J Respir Cell Mol Biol. 2009; 40(5):519–35. doi: 10.1165/rcmb.2008-0348TR PMID: 18978300

64. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. Nat Rev Immunol. 2013; 13(3):159–75. doi: 10.1038/nri3399 PMID: 23435331

65. Caudrillier A, Kessenbrock K, Gilliss BM, Nguyen JX, Marques MB, Monestier M, et al. Platelets induce neutrophil extracellular traps in transfusion-related acute lung injury. J Clin Invest. 2012; 122(7):2661–71. doi: 10.1172/JCI61303 PMID: 22684106

66. Cheng OZ, Palaniyar N. NET balancing: a problem in inflammatory lung diseases. Front Immunol. 2013; 4:1. doi: 10.3389/fimmu.2013.00001 PMID: 23355837

67. Yeh DY, Kao SJ, Feng NH, Chen HI, Wang D. Increased nitric oxide production accompanies blunted hypoxic pulmonary vasoconstriction in hyperoxic rat lung. Chin J Physiol. 2006; 49(6):305–12. PMID: 17357537

68. Ulrich R, Bloch KD, Ichinose F, Steudel W, Zapol WM. Hypoxic pulmonary blood flow redistribution and arterial oxygenation in endotoxin-challenged NOS2-deficient mice. J Clin Invest. 1999; 104(10):1421–9. PMID: 10562304

69. Spöhr F, Cornelissen AJ, Busch C, Gebhard MM, Motisch J, Martin EO, et al. Role of endogenous nitric oxide in endotoxin-induced alteration of hypoxic pulmonary vasoconstriction in mice. Am J Physiol Heart Circ Physiol. 2005; 289(2):H823–31. PMID: 15778287

70. Revollo JR, Kümer A, Mills KF, Satoh A, Wang T, Garten A, et al. Nampt/PBEF/Visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. Cell Metab. 2007; 6(5):363–75. PMID: 17983582