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DISSERTATION

Protektiver Effekt einer pharmakologischen Aktivierung des Nrf2-ARE Signalweges in einem experimentellen Modell des beatmungs-induzierten Lungenschadens/ Pharmacological activation of the Nrf2-ARE pathway and its protective effects in an experimental model of ventilator-induced lung injury

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1. Abbreviations

ARDS	acute respiratory distress syndrome
ARE	antioxidant response element
BALF	broncho-alveolar lavage fluid
EGCG	epigallocatechin-3-gallate
EtOH3%	3% ethanol
GSH	glutathione (reduced glutathione)
Gclc	glutamate-cysteine ligase catalytic subunit
Gclm	glutamate-cysteine ligase modifier subunit
Gpx2	glutathione peroxidase 2 (gene)
GPX2	glutathione peroxidase 2 (protein)
GSSG	glutathione disulfide (oxidized glutathione)
HV_T	high tidal volume
IL-1β	interleukin 1 beta
IL-6	interleukin 6
Keap1	Kelch-like ECH-associated protein 1
LPS	lipopolysaccharide
LV_T	low tidal volume
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
Nqo1	NAD(P)H dehydrogenase [quinone] 1 (gene)
NQO1	NAD(P)H dehydrogenase [quinone] 1 (protein)
Nrf2	nuclear factor erythroid 2-related factor 2 (gene)
NRF2	nuclear factor erythroid 2-related factor 2 (protein)
PEEP	positive end-expiratory pressure
qPCR	quantitative polymerase chain reaction
Sod1	superoxide dismutase 1
tBHQ	*tert*-butylhydroquinone
TNF-α	tumor necrosis factor alpha
VILI	ventilator-induced lung injury

2. Abstract

Ventilator-induced lung injury (VILI) is a serious complication of mechanical ventilation. A contributing factor to the pathophysiology of VILI is oxidative stress. A physiological protective response to oxidative stress is the activation of the Nrf2-ARE pathway. This induces Nrf2-dependent gene expression, ultimately increasing the production of antioxidant proteins to restore redox balance. Pharmacological activation of the Nrf2-ARE pathway is possible with Nrf2 activators including *tert*-butylhydroquinone (tBHQ) and epigallocatechin-3-gallate (EGCG). The aim of this project was to test whether pharmacological activation of the Nrf-ARE pathway with tBHQ or EGCG is protective against VILI.

A mouse model of VILI using high tidal volume (HV_T) ventilation to cause acute lung injury was used to answer this question. Prior to mechanical ventilation mice were pretreated with tBHQ, EGCG, 3% ethanol (EtOH3%, vehicle for tBHQ), or phosphate buffered saline (controls). Survival, arterial blood oxygenation and respiratory system compliance were measured to assess functional outcome. Nrf2-dependent gene expression and antioxidant proteins (glutathione, NRF2) were used as surrogates to evaluate the activation the Nrf2-ARE pathway.

HV_T ventilation severely impaired arterial blood oxygenation and respiratory system compliance, resulting in 100% mortality among controls. Pretreatment with tBHQ improved survival (60%, p<0,001), arterial blood oxygenation and respiratory system compliance compared with controls. Pretreatment with tBHQ also reduced HV_T ventilation-associated increase of pro-inflammatory cytokines and increased pulmonary redox capacity indicated by increased pulmonary Nrf2-dependent relative gene expression of *Nrf2* and *Sod1* at baseline as well as increased concentration of antioxidant proteins NRF2 and glutathione after HV_T ventilation. Compared with controls, pretreatment with EtOH3% also improved survival (38,5%, p=0,005) and reduced the pro-inflammatory response in the lung, but did not increase pulmonary redox capacity. Pretreatment with EGCG only minimally improved survival (20%, p=0,061), but neither arterial blood oxygenation nor respiratory system compliance showed an improvement. Pretreatment with EGCG did not reduce HV_T ventilation-associated pro-inflammatory response, but increased the relative expression of *Gpx2* and *Nqo1* at baseline and yielded in the highest relative expression of *Gpx2* after HV_T ventilation.

In this mouse model of VILI, tBHQ increases the pulmonary redox capacity by activating the Nrf2-ARE pathway and protects against VILI. The protective effect of EtOH3% may be mediated by an attenuation of inflammation independent of Nrf2. Despite slightly increasing the Nrf2-dependent gene expression EGCG did not provide any relevant protection against VILI.

3. Abstract (german version)

Eine schwerwiegende Komplikation der invasiven Beatmung ist der beatmungsinduzierte Lungenschaden (VILI). Oxidativer stress spielt eine wichtige Rolle in der Pathophysiologie des VILI. Eine physiologische Schutzreaktion auf oxidativen Stress ist die Aktivierung des Nrf2-ARE-Signalwegs. Dieser induziert im Falle von oxidativem Stress eine Nrf2-abhängige Genexpression und erhöht letztendlich die Produktion von antioxidativen Proteinen, um das Redoxgleichgewicht wiederherzustellen. Eine pharmakologische Aktivierung des Nrf2-ARE Signalweges ist mit sogenannten Nrf2-Aktivatoren wie *tert*-butylhydroquinone (tBHQ) oder epigallocatechin-3-gallate (EGCG) möglich. Ziel dieses Forschungsprojekts war es zu testen, ob die pharmakologische Aktivierung des Nrf2-ARE Signalweges mit tBHQ oder EGCG eine Protektion gegen VILI darstellt.

Dazu wurde ein Mausmodell verwendet, bei dem durch Beatmung mit einem hohen Tidalvolumen (HV_T) ein beatmungsinduzierter Lungenschaden verursacht wurde. Die Mäuse wurden vor dem Beginn der Beatmung mit tBHQ, EGCG, 3% Ethanol (EtOH3%, Trägersubstanz für tBHQ) oder posphatgepufferte Kochsalzlösung (Kontrollgruppe) vorbehandelt. Die Überlebensrate, der arterielle Sauerstoffpartialdruck und die Compliance des Atemapparates wurden gemessen um das funktionelle Outcome zu bewerten. Nrf2-abhängige Geneexpression und anti-oxidative Proteine (NRF2, glutathione) wurden als Surrogatparameter für die Aktivierung des Nrf-ARE Signalweges verwendet.

HV_T-Beatmung beeinträchtigte die arterielle Oxygenierung und die Compliance des Atemapparates erheblich, so dass eine 100%ige Mortalität der Kontrollen resultierte. Die Vorbehandlung mit tBHQ verbesserte die Überlebensrate (60%, $p<0,001$), die arterielle Oxygenierung und die Compliance des Atemapparates im Vergleich zu den Kontrollen. Die Vorbehandlung mit tBHQ reduzierte auch die mit der HV_T-Beatmung assoziierte Zunahme der pro-inflammatorischer Zytokine und erhöhte ebenso die pulmonale Redox-Kapazität: die pulmonale Nrf2-abhängige relative Genexpression von *Nrf2* und *Sod1* vor Beginn der Beatmung sowie die Konsentration antioxidativer Proteine (NRF2, Glutathion) nach HV_T-Beatmung waren erhöht. Die Vorbehandlung mit EtOH3% verbesserte ebenfalls die Überlebensrate (38,5%, $p=0,005$) und verringerte die pro-inflammatorische Reaktion in der Lunge, erhöhte jedoch nicht die pulmonale Redoxkapazität. Die Vorbehandlung mit EGCG verbesserte die

Überlebensrate nur minimal (20%, p=0,061), verbesserte jedoch weder die arterielle Oxygenierung noch die Compliance des Atemapparates. Die Vorbehandlung mit EGCG reduzierte zwar nicht die Entzündungsreaktion, erhöhte aber die relative Genexpression von *Gpx2* und *Nqo1* zu Beginn der Beatmung und ergab nach HV_T-Beatmung die höchste relative Expression von *Gpx2.*

In diesem Mausmodell des VILI erhöht tBHQ die pulmonale Redoxkapazität durch Aktivierung des Nrf2-ARE-Signalwegs und zeigt eine protektive Wirkung gegen VILI. Der protektive Effekt von EtOH3% scheint durch eine von Nrf2 unabhängige Inflammationshemmung vermittelt zu werden. Trotz geringfügiger Erhöhung der Nrf2-abhängigen Genexpression bietet EGCG keinen relevanten Schutz gegen VILI.

4. Background

Mechanical ventilation is a modality widely used in modern medicine whenever the respiratory system fails to sufficiently provide the body with oxygen or remove carbon dioxide. It replaces the work otherwise done by respiratory muscles that provide energy to move air into the lungs (30). The history of mechanical ventilation dates back to the early 19th century, when first negative pressure ventilators were invented, yet its full clinical value became obvious more than a century later during the polio pandemic (67). Consequently, the clinical use of mechanical ventilation increased. In the second half of 20th century positive pressure ventilation that was able to sustain higher airway pressures and establish positive end-expiratory pressure (PEEP) to improve gas exchange marginalized the use of negative pressure ventilation (37). Today, mechanical ventilation is used daily during general anaesthesia, when respiratory failure is a side effect of drugs that affect the respiratory centre in the brain (opioids, sedatives) or paralyse the respiratory muscles (muscle relaxants). Mechanical ventilation is also often required when respiratory failure occurs due to pathologies affecting the respiratory system, like neuromuscular disease causing malfunctioning of the respiratory muscles, bronchial asthma causing the obstruction of airways, pulmonary embolism blocking the pulmonary circulation, or diseases directly affecting the lung parenchyma such as pneumonia.

4.1. Basic principles of pulmonary gas exchange

Gas exchange takes place in the lungs, more precisely in the alveoli. Alveoli are delicate sacs mainly comprised of type I and type II pneumocytes as well as resident alveolar macrophages. Type I pneumocytes that are thin and flat squamous cells, cover most of the alveolar surface creating a fine barrier for gas exchange (8). The large and cuboidal type II pneumocytes have two major functions: production of surfactant that lowers pulmonary surface tension to prevent alveolar collapse and repair of the alveolar membrane by differentiating into type I pneumocytes (8, 22). Together with the mesh of pulmonary capillaries around the alveolar membrane, alveoli form a gas exchange unit where a pressure gradient across the alveolar-endothelial barrier allows oxygen to diffuse into the blood stream and carbon dioxide into the alveoli.

The role of ventilation is to transport oxygen into the alveoli and remove carbon dioxide from it in order to maintain the necessary pressure gradient across the alveolar-endothelial barrier for pulmonary gas exchange. Similarly the blood flow in the pulmonary capillaries surrounding the alveoli needs to be uninterrupted in order to create a pressure gradient across the alveolar-endothelial barrier. In summary, gas exchange is compromised whenever air cannot freely enter and exit the alveoli, like in case of alveolar collapse or edema, whenever the alveolar-endothelial barrier – the diffusion membrane – is thickened like in the case of interstitial edema or fibrosis, and whenever the perfusion of the pulmonary capillaries is impaired or blocked like during pulmonary embolism. In any of these aforementioned cases a ventilation-perfusion mismatch occurs creating a shunt that allows poorly oxygenated and decarboxylated blood flow back in to the systemic blood stream.

4.2. Ventilator-induced lung injury

Concerns about the safety of mechanical ventilation emerged when it was increasingly used to help patients with lung disease, who often required invasive ventilation with high airway pressure and volume in order to maintain sufficient gas exchange (67). Mechanical forces applied onto the lung surface during mechanical ventilation such as excessive pressure and volume can cause alveolar overdistension and in a worst-case scenario literally tear the lung parenchyma, hence causing pneumothorax. Pneumothorax was not an uncommon complication of mechanical ventilation in the early days when the main focus was on maintaining normal blood gases (26). In addition, gradually evidence emerged about the beneficial effects of an optimal PEEP although the concerns about its negative hemodynamic effects prevailed for years (5, 28, 72). In 1993 Parker et. al. introduced the term ventilator-induced lung injury (VILI) and summarized its main mechanisms (baro-, volu- and atelectrauma) and risk factors (56, 81). VILI, a serious complication of mechanical ventilation, can contribute to the lung damage that was the indication for mechanical ventilation in the first place, creating a vicious cycle.

4.3. Pathophysiology of acute respiratory distress syndrome

A particular challenge is providing ventilatory support to patients with acute respiratory distress syndrome (ARDS). ARDS, first recognized in 1967, is a serious lung condition characterized by sudden onset non-cardiac pulmonary edema causing hypoxemia that according to the Berlin Definition of ARDS can be mild (200 mmHg < PaO_2/FIO_2 ≤ 300 mmHg), moderate (100 mm Hg < PaO_2/FIO_2 ≤ 200 mm Hg), or severe (PaO_2/FIO_2 ≤ 100 mm Hg) (5, 23). ARDS is a severe form of lung damage, histologically characterized by diffuse alveolar damage (DAD), with either pulmonary (e.g. pneumonia, aspiration, pulmonary embolism) or extra-pulmonary (e.g. sepsis, polytrauma, severe burns) etiology (14).

The basic pathophysiological features include pulmonary inflammation, disruption of alveolar-endothelial barrier, interstitial and intra-alveolar lung edema. The development of alveolar injury can be divided into three sequential phases — exudative, proliferative and fibrotic — that overlap considerably. Exudative phase is characterized by immune-cell (neutrophils, macrophages) mediated inflammatory destruction of the alveolar-endothelial barrier, allowing the influx of protein-rich inflammatory exudate into the interstitial and alveolar space (73). The damage to the alveolar epithelium also impairs the clearance of edema fluid and leads to surfactant depletion. Endothelial damage causes endothelial dysfunction that not only worsens ventilation-perfusion mismatch, but also increases pulmonary arterial pressure and contributes to the development of right heart failure (43). The proliferative phase that is characterized by the proliferation of type II pneumocytes and their differentiation into type I pneumocytes is an attempt to restore the alveolar membrane (73). The fibrotic phase that can run in parallel to the exudative and proliferative phase is characterized by up-regulation of collagen synthesis leading to progressive fibrotic changes in any part of the gas exchange unit (13). Histologically, DAD is characterized by edema, hyaline membranes, and interstitial acute inflammation in the acute (exudative) phase, and by fibrosis and type II pneumocyte hyperplasia in the subacute (organizing) phase (14).

4.4. Role of VILI in ARDS patients

Lungs of ARDS patients are especially susceptible for damage from mechanical ventilation due to several factors. Not only is the severely damaged lung more fragile, but the fluid-filled and consolidated alveoli considerably reduce the functional lung capacity ("baby lung concept") that can be mechanically ventilated (28). This makes the ventilated alveoli more prone to overdistension. Moreover, the lung damage is usually in-homogenous, meaning that next to the aveoli that can be ventilated there are aveoli that are consolidated, collapsed or filled with edema fluid (30). This can massively increase the stress and strain placed on the alveoli that are ventilated.

Mechanical ventilation that plays a central role in the management of ARDS was long suspected to contribute to the lung damage and even to the mortality of ARDS patients. But it was not until the landmark study from the ARDS-Network was published showing that the use of lower tidal volumes improves survival of ARDS patients, when the importance of VILI was finally proved and gained real clinical significance (10). Today, protective ventilatory strategies, such as the use of low tidal volumes (4-6 ml/kg), appropriate PEEP settings (ARDS-Network table), and limiting plateau airway pressure and driving pressure have become common clinical practices for ventilating patients with ARDS to limit VILI (4, 9, 10, 33). Mechanical ventilation in prone position can also limit the development of VILI and reduce the mortality of patients with ARDS (34, 52). A complex interplay of gravitational forces, specifics of lung anatomy and change in respiratory system mechanics in prone position not only improves gas exchange by reducing ventilation-perfusion mismatch, but also reduces stress and strain on the ventilated lung areas (27, 62). It is a recommended therapy for patients with moderate to severe ARDS (33). Nevertheless, mortality among patients with ARDS remains high (7, 21).

4.5. Biotrauma concept in VILI

Even though mechanical injury of the lung from excessive pressure (barotrauma) and tidal volume (volutrauma) as well as repetitive opening and closing of the lung (atelectrauma) is preventable with lung-protective ventilation strategies, a more subtle biochemical injury still occurs (25, 29). It has been shown that just stretching of the lung tissue (alveolar macrophages, epithelial and endothelial cells) without the

disruption of lung parenchyma induces the production of pro-inflammatory cytokines and reactive oxygen/nitrogen species (ROS/RNS) (15, 36, 49, 59). The term biotrauma that summarizes this cellular biochemical response to mechanical stretching was first described in 1998 by Slutsky and colleagues (19, 82). The significance of biotrauma lies within the possibility that it can contribute to multiple organ failure and death of critically ill patients. The initial flooding of lung with biochemical mediators can progress and lead to systemic inflammation and oxidative stress that can damage various organs (19).

Interestingly, most patients with ARDS do not die from their pulmonary disease but rather from multiple organ failure. Studies have shown that persistent elevation of pro-inflammatory cytokines in broncho-alveolar lavage fluid (BALF) and in the plasma are associated with a poor outcome in patients with ARDS (46, 47). Moreover, in the ARDS-Network study showing the protective effect of lower tidal volumes, patients in the lower tidal volume group also had lower levels of pro-inflammatory cytokines (IL-6, IL-8, TNF-α) in the plasma on the first three days of mechanical ventilation that might have contributed to the improved survival (10). This indicates that the level of systemic inflammation not only plays a role in the mortality of ARDS patients but VILI is a relevant contributing factor to it.

Oxidative stress that contributes to the pathophysiology of VILI is also present in patients with ARDS. Glutathione, a major antioxidant, was more prevalent in the oxidized from in the lungs of patient with ARDS indicating pulmonary oxidative stress (12). Moreover, plasma concentrations of lipid peroxidation products such as malondialdehyde (MDA) were increased and the levels of endogenous antioxidants were decreased in patients with ARDS, indicating systemic oxidative stress (48). Proper redox balance seems to be crucial to maintain the integrity and functionality of lung cells and thus reducing the susceptibility to VILI (61). Of note, oxidative stress also promotes inflammation.

4.6. Antioxidant Nrf2-ARE pathway

As the maintenance of redox balance is crucial to the integrity and functionality of all cell types, it is not surprising that there are sophisticated mechanisms to counteract oxidative stress. First line defence against oxidative stress are complex systems

comprised of antioxidants (e.g. glutathione, vitamin C) and enzymes (e.g. glutathione peroxidase, NAD(P)H dehydrogenase [quinone], superoxide dismutase) to limit the production and reduce the concentration of ROS/RNS. However, oxidative stress can also exceed the available cellular redox capacity. To avoid the possible scarcity of antioxidant resources, a physiological response to oxidative stress is the induction of antioxidant gene expression. This ultimately leads to the increased production of various enzymes that are involved either in the synthesis of antioxidants or in the chemical reactions that reduce the levels of ROS/RNS. In mammalian cells this process is regulated by nuclear factor-erythroid-2-related factor 2 (Nrf2) (80). Nrf2 is a transcription factor that can initiate the transcription of many antioxidant and cytoprotective genes (80). This process is tightly regulated in the cells. Under non-stress conditions Nrf2 binds to its cytoplasmatic inhibitor Kelch-like ECH-associated protein 1 (Keap1) that allows the ubiquitylation of Nrf2 leading to its proteosomal degradation (11). Keap1 is redox-sensitive meaning that under oxidative stress Nrf2 is released. Free Nrf2 can translocate to the nucleus and bind to the antioxidant response element (ARE) in the upstream promoter region of many antioxidant and cytoprotective genes (11). The relevance of the Nrf2-ARE pathway in the cellular protection underscores the fact that many cancer cells hijack this pathway thus increasing cell survival to radio- and chemotherapy (77).

Knowing that mechanical ventilation induces oxidative stress, it is not surprising that a physiological response to mechanical ventilation is the up-regulation of Nrf2-dependent gene expression (54). The level of oxidative stress as well as the antioxidant response is dependent on the level of stretch and strain (70). It is noteworthy that mice genetically deficient of *Nrf2* develop more severe lung injury from mechanical ventilation (55).

4.7. Pharmacological activation of the Nrf2-ARE pathway

Pharmacological activation of the Nrf2-ARE pathway with specific activators such as *tert*-butylhydroquinone (tBHQ) and epigallocatechin-3-gallate (EGCG) is also possible (11, 71). tBHQ is a food preservative (named E319) that prevents oxidation of vegetable oils and many edible animal fats (max. level allowed in fat products is 0,02%). It has been shown that by attenuating oxidative stress, tBHQ protected the

kidney and liver against ischemia-reperfusion injury in rats (35, 74). In addition, pharmacological activation of the Nrf2-ARE pathway with tBHQ reduced mortality and severity of lung damage in mice with lipopolysaccharide (LPS)-induced lung injury (85).

EGCG is a polyphenolic catechin found in green tea. EGCG has been shown to increase the expression of Nrf2-dependent antioxidant genes and to reduce the expression of pro-inflammatory cytokines in human lung cells *in vitro* (17, 78). EGCG has also been shown to have an anti-inflammatory effect and provide protection against bleomycin-induced lung injury in rats, as well as oleic acid- or LPS-induced lung injury in mice (6, 68, 87). Anti-inflammatory and antioxidant effects of EGCG have also been shown in models of intestinal inflammation as well as ischemia-reperfusion animal models (1, 31)

The aim of this doctoral thesis was to determine whether pharmacological activation of the Nrf2-ARE pathway with tBHQ or EGCG prior to mechanical ventilation increases cellular redox capacity and provides protection against VILI.

5. Methods

5.1. Experimental protocol

A mouse model of VILI using high tidal volume (HV_T) ventilation to induce acute lung injury was established to answer the above mentioned research question. A one hit model was used, meaning that injurious mechanical ventilation was the sole cause of acute lung injury.

Eight to ten week-old male C57BL/6 mice received a single intra-peritoneal (i.p.) injection of tBHQ, EGCG, 3% ethanol (EtOH3%, vehicle for tBHQ), or phosphate buffered saline (PBS, controls). One hour later, general anaesthesia was induced with an i.p. bolus of ketamine and xylazine. Mice were then tracheostomised and subjected to low tidal volume (LV_T) ventilation (10ml/kg). Mice received a carotid catheter for hemodynamic monitoring as well as continuous administration of fluids and anaesthetics to avoid strong cardio-circulatory effects from volume overload and boluses of anaesthetics. Throughout mechanical ventilation mice were paralysed with pancuronium in order to avoid any lung damage from asynchronous spontaneous breathing.

One hour after initiation of LV_T ventilation mice were subjected to HV_T ventilation (40-42,5 ml/kg) for maximum of four hours or until the mean arterial blood pressure had dropped below 40 mmHg for more than 10 seconds. Thus, survival, one of the main outcomes, was defined as mean arterial pressure $\geq$ 40 mmHg at four hours of HV_T ventilation. In line with other authors, in this mouse model of VILI mean arterial blood pressure $<$ 40 mmHg accurately predicted imminent death and was used to define mortality (51). In order to cause lethal lung injury to an otherwise healthy mouse lung (one hit model), mechanical ventilation with tidal volumes that surpassed the total lung capacity were necessary (58, 79). Other functional outcomes to assess severity of VILI were respiratory system compliance (dynamic and semistatic) as well as arterial blood oxygenation.

At the end of the experiments bronchoalveolar lavage was performed and lungs were harvested. All material was snap frozen in liquid nitrogen for further analysis. Pro-inflammatory cytokines in BALF were measured to assess pro-inflammatory response to HV_T ventilation. Also, total protein concentration in BALF was used as a surrogate

to assess the level of pulmonary edema. Nrf2-dependent gene expression and levels of antioxidant proteins (glutathione, NRF2) were measured prior to mechanical ventilation (baseline) and after HV_T ventilation to assess the activation of the Nrf2-ARE pathway physiologically to mechanical ventilation and pharmacologically to tBHQ and EGCG.

In order to measure baseline activation of the Nrf2-ARE pathway, separate groups of mice were pretreated with tBHQ, EGCG, EtOH3%, or PBS and sacrificed one hour later without being subjected to mechanical ventilation. Additionally, a separate group of mice were only subjected to LV_T ventilation for five hours to assess the pro-inflammatory reaction and level of pulmonary edema to non-injurious mechanical ventilation.

All details concerning the mouse model of VILI, biochemical measurements and statistical analysis as well as experimental design involving tBHQ can be found in the supplemental digital content of a previous publication (https://doi.org/10.6084/m9.figshare.12895790) (83).

5.2. Experimental specifics for EGCG

A stock solution was prepared for epigallocatechin-3-gallate (EGCG, Sigma-Aldrich, USA) by dissolving 50mg EGCG in 11,1 ml of saline yielding a final concentration of 4,5 mg/ml (maximum water-solubility 10mM). As EGCG stability is higher at higher concentration, lower pH and lower temperature, the stock solution was divided into 0,3 ml aliquots and kept in the freezer (-20C) until further use (39, 57). Just before usage 0,3 ml of EGCG 4,5 mg/ml solution was thawed and diluted with 1,5 ml of saline yielding in a final concentration of 0,75 mg/ml. The pH was adjusted to 7,4 with 0,1M NaOH.

C57BL/6J mice were randomly assigned to receive a single i.p. injection of EGCG (5 mg/kg, n=10) or equivalent volume PBS (controls, n=10) one hour before anaesthesia induction. To find the optimal dosage for EGCG, mice received a single i.p. injection of EGCG (5, 10, or 50 mg/kg) or equivalent volume of PBS, one hour prior to being anaesthetised and sacrificed to analyse Nrf2-dependent antioxidant gene expression in the lung tissue.

All physiological and biochemical measurements as well as statistical analysis were done the same way as described in the supplemental digital content of our previous publication (https://doi.org/10.6084/m9.figshare.12895790) (83). Only the use of TaqMan probes (Applied Biosystems, USA) *Gpx2* (Mm00850074_g1) and *Nqo1* (Mm01253561_m1) were not included there.

5.3. Modelling acute lung injury: strengths and weaknesses

The main features of acute lung injury in an animal model are the damage of alveolar-endothelial barrier, an inflammatory reaction, physiological dysfunction and histologic evidence of tissue injury (44). In this project we measured three out of the four hallmarks to assess the severity of acute lung injury. Total protein concentration in BALF was used as a surrogate to assess the permeability of alveolar-endothelial barrier. Concentrations of pro-inflammatory cytokines in BALF were measured to assess the inflammatory response. Arterial blood oxygenation was used to assess the level of hypoxemia as a marker for physiological dysfunction. All three measurements — total protein concentration in BALF, pro-inflammatory cytokines in lung tissue or BALF, and hypoxemia — are considered very relevant in the assessment of acute lung injury (44).

The use of extremely high tidal volumes does not necessarily reduce the translational relevance of this mouse model of VILI. Firstly, in the clinical setting most often VILI becomes relevant in the setting of already present inflammatory process such as ARDS. Experimental data show that even moderate tidal volumes can cause acute lung injury in the presence of inflammation (2, 3, 53). Secondly, in patients with ARDS who have considerably reduced total lung capacity (“baby lung concept”) and high lung inhomogeneity, local overdistension occurs even when using lower tidal volumes (30).

The advantage of this model was that it let us concentrate solely on the effect of injurious mechanical ventilation. The widespread use of mechanical ventilation in clinical practice also makes it more relevant compared with models using hyperoxia, acid aspiration, oleic acid or bleomycin (45). The main disadvantage was that the model was fairly complex requiring surgical skills (tracheostomy, catheterisation of carotid artery) as well as advanced monitoring (mean arterial blood pressure, pulse rate, tidal volume, peak inspiratory pressure and PEEP). Also, the scarcity of

expensive equipment and complexity of the model made it possible to perform only one experiment per day.

6. Results

6.1. Effects of HV_T ventilation

HV_T ventilation caused 100% mortality among controls compared with LV_T ventilation that caused 0% mortality (p<0,001, log-rank Mantel-Cox test). HV_T ventilation severely impaired arterial blood oxygenation (Figure 2, PaO_2 49 ± 7 mmHg) compared with LV_T ventilation (Figure 2, PaO_2 209 ± 14 mmHg, p<0,001, unpaired Student's t-test) and increased the total concentration of protein (Figure 3, LV_T vs HV_T p<0,001, unpaired Student's t-test) in BALF.

HV_T ventilation caused a significant reduction of respiratory system compliance: dynamic compliance decreased from 34 ± 1,7 µl/cm H_2O to 22 ± 1.3 µl/cm H_2O (~35%) (Table 2, p<0,001, two-way repeated measures ANOVA) and semistatic compliance from 57 ± 4,1 µl/cm H_2O to 29 ± 2.7 µl/cm H_2O (~50%) (Table 2, p<0,001, two-way repeated measures ANOVA) after HV_T ventilation.

HV_T ventilation also increased pro-inflammatory cytokines IL-1β (Figure 4A, p<0,001, unpaired Student's t-test) and TNF-α (Figure 4B, p<0,001, Mann-Whitney U-test) in BALF that was associated with the activation of the canonical pathway of nuclear factor kappa B (NFκB) (Figure 5, p=0,003, unpaired Student's t-test).

HV_T ventilation per se increased the relative pulmonary expression of all measured Nrf2-dependent genes: *Nrf2* (Figure 6A, p<0,001, unpaired Student's t-test), *Sod1* (Figure 6B, p<0,001, Mann-Whitney U-test), *Gclc* (Figure 6C, p<0,001, Mann-Whitney U-test), *Gclm* (Figure 6D, p<0,001, Mann-Whitney U-test), *Gpx2* (Figure 10B, p<0,001, Mann-Whitney U-test), *Nqo1* (Figure 10C, p<0,001, unpaired Student's t-test).

HV_T ventilation also increased the concentration of NRF2 protein from 14 ± 3,1 pg/ml to 21 ± 4,1 pg/ml (Figure 7A, p<0,001, unpaired Student's t-test), total concentration of glutathione from 28 ± 5,4 mmol/g to 36 ± 4,5 mmol/g tissue (Figure 7B, p=0,011, unpaired Student's t-test) and GSH/GSSH ratio from 5 ± 2,5 to 13 ± 3,6 (Figure 8, p<0,001, unpaired Student's t-test) in lung tissue.

6.2. Stability of the mouse model of VILI

At the beginning of HV_T ventilation there were no differences between hemodynamic parameters such as pulse rate and blood pressure as well as ventilatory parameters such as peak inspiratory pressure and tidal volume between all groups (Table 1). Respiratory system compliance, dynamic and semistatic, was also comparable in all groups before HV_T ventilation (Table 2, Table 3).

	controls	EtOH3%	tBHQ	EGCG
Weight (g)	23,9 ± 1,1	24,8 ± 1,0	24,9 ± 0,7	24,9 ± 1,3
Pulse rate (bpm)	390 ± 21	400 ± 42	410 ± 32	390 ± 20
Mean arterial blood pressure (mmHg)	73 ± 8,6	77 ± 8,2	75 ± 5,7	78 ± 7,8
Peak inspiratory pressure (cmH_2O)	30 ± 0,4	30 ± 0,3	30 ± 0,2	30 ± 0,4
Tidal volume (ml/kg)	41 ± 1,0	41 ± 0,9	41 ± 0,7	41 ± 1,0

Tabel 1. Basic parameters at the beginning of HV_T ventilation. Mice were pretreated with PBS (controls, n=10), EtOH3% (n=13), tBHQ (n=10) or EGCG (n=10), and subjected to HV_T ventilation. Mean arterial blood pressure (mmHg), pulse rate (bpm), peak inspiratory pressure (cmH_2O) and tidal volume (ml/kg) were measured at the beginning of HV_T ventilation.

6.3. tBHQ: Survival and arterial blood oxygenation

Compared with controls, pretreatment with tBHQ resulted in the survival of 60% of mice at 4 hours of HV_T ventilation (Figure 1, $p<0,001$, log-rank Mantel-Cox test). Improved survival with tBHQ was associated with better arterial blood oxygenation at the end of HV_T ventilation compared with controls (Figure 2, PaO2 90 ± 41 mmHg, $p=0,018$, Kruskal-Wallis test).

EtOH3% also improved survival (Figure 1, 38,5%, $p=0,005$, log-rank Mantel-Cox test), but did not improve arterial blood oxygenation at the end of HV_T ventilation compared with controls (Figure 2, PaO2 of 73 ± 42 mmHg, $p=0,813$, Kruskal-Wallis test).

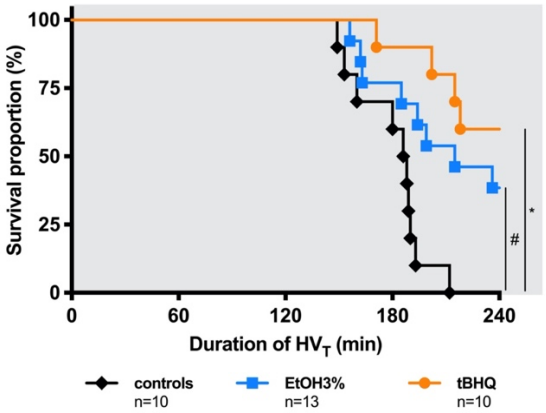

Figure 1. tBHQ: survival of mice subjected to HV_T ventilation. Mice were pretreated with PBS (controls, n=10), EtOH3% (n=13), or tBHQ (n=10), and then subjected to HV_T ventilation. Mice were sacrificed when the mean arterial pressure decreased to less than 40 mmHg or after a maximum duration of 4 hours of HV_T ventilation. *p<0,05 tBHQ versus controls, #p<0,05 EtOH3% versus controls. Adapted from Veskemaa et. al. (83).

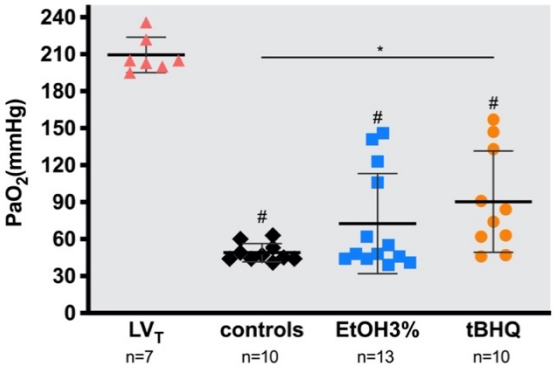

Figure 2. tBHQ: arterial blood oxygen (PaO_2) tension of mice subjected to HV_T ventilation. Mice were pretreated with PBS (controls, n=10), EtOH3% (n=13), or tBHQ (n=10), and then subjected to HV_T ventilation. A separate group of mice were pretreated with PBS and subjected to LV_T ventilation for 5 hours (LV_T, n=7). PaO_2 was measured in arterial blood obtained from the carotid artery at the end of the experiment. The inspired oxygen fraction was 0,4 in all groups. *<0,05 tBHQ versus controls . #p<0.05 LV_T versus HV_T. Adapted from Veskemaa et. al. (83).

6.4. tBHQ: lung edema and respiratory mechanics

Total protein concentration in BALF was used as a surrogate to assess the degree of lung edema. Pretreatment with tBHQ resulted in a 35% lower total protein concentration in BALF (Figure 3, 1,3 ± 0,6 µg/µl, p=0,093, one-way ANOVA) compared with controls (Figure 3, 2,2 ± 0,7 µg/µl). Pretreatment with tBHQ reduced the HV_T ventilation-associated decrease of dynamic (Table 2, 28 ± 5,1 µl/cm H_2O, p=0,040, one-way ANOVA) and semistatic respiratory system compliance (Table 2, 37 ± 9,0 µl/cm H_2O, p=0,009, one-way ANOVA) compared with controls.

Compared with controls, pretreatment with EtOH3% did not influence the degree of lung edema (Figure 3, 1,9 ± 0,6 µg/µl, p>0,999, one-way ANOVA) and resulted in a similar reduction of dynamic (Table 2, 26 ± 4,4 µl/cm H_2O, p=0,114, one-way ANOVA) and semistatic respiratory system compliance (Table 2, 32 ± 6,1 µl/cm H_2O,, p>0,999, one-way NOVA).

	Dynamic Respiratory System Compliance (µl/cm H_2O)		Semistatic Respiratory System Compliance (µl/cm H_2O))	
	Before HV_T	After HV_T	Before HV_T	After HV_T
controls	34 ± 1,7	22 ± 1,3#	57 ± 4,1	29 ± 2,7#
EtOH3%	35 ± 1,8	26 ± 4,4#	60 ± 3,3	32 ± 6,1#
tBHQ	35 ± 1,5	28 ± 5,1*#	60 ± 3,0	37 ± 9,0*#

Table 2. tBHQ: respiratory system mechanics. Respiratory system mechanics measured before the initiation HV_T ventilation (before HV_T) and after HV_T ventilation (after HV_T) pretreated with PBS (controls, n≥ 9), EtOH3% (n=13), or tBHQ (n≥ 9). All values presented mean±SD. *p<0,05 tBHQ after HV_T versus controls after HV_T, #p<0,05 before HV_T versus after HV_T. Missing samples: 1 control and 1 tBHQ for semistatic compliance (technical failure to record pressure-volume curves). Adapted from Veskemaa et. al. (83).

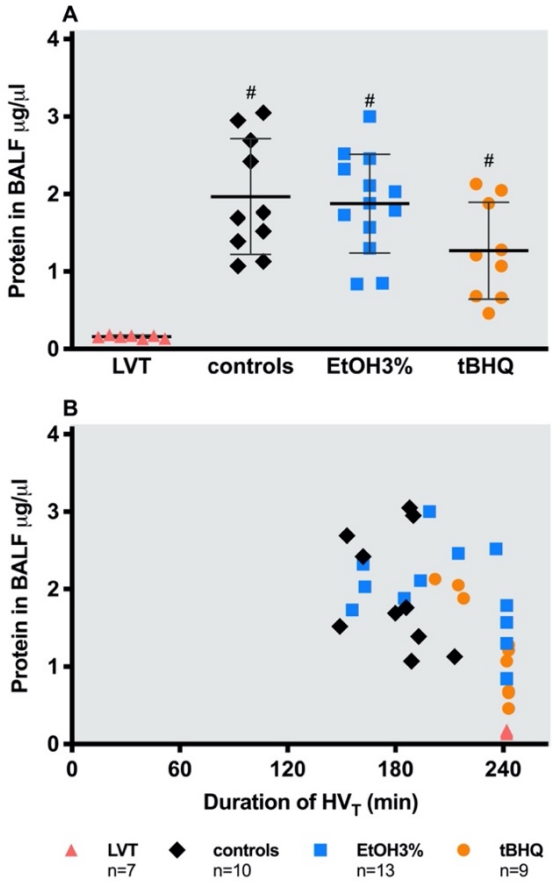

Figure 3. tBHQ: total concentration of protein in BALF of mice subjected to HV_T ventilation. Mice were pretreated with PBS (controls, n=10), EtOH3% (n=13), or tBHQ, (n=9), and then subjected to HV_T ventilation. A separate group of mice were pretreated with PBS and subjected to LV_T ventilation for 5 hours (LV_T, n=7). As a surrogate for pulmonary edema, total protein concentration was measured in the BALF obtained after LV_T and HV_T ventilation (A). #p<0,05 LV_T versus HV_T (unpaired Student's t-test). Missing samples: 1 tBHQ (failure to collect BALF). A scatter plot (B) was created to depict possible correlations between the total concentration of protein in BALF and the duration of HV_T ventilation. Only in the tBHQ subgroup a strong negative correlation (r=-0.892, p<0,05, Pearson correlation) was found. Adapted from Veskemaa et. al. (83).

6.5. tBHQ: Inflammation in the lung

Compared with controls pretreatment with tBHQ decreased the concentrations of pro-inflammatory cytokines IL-1β (Figure 4A, p=0,030, Kruskal-Wallis test) and TNF-α in BALF (Figure 4B, p=0,025, Kruskal-Wallis). Also, compared with controls pretreatment with tBHQ decreased the activation of the inflammatory canonical pathway of nuclear factor kappa B (NF-κB), indicated by decreased phosphorylated p65/total p65 ratio (Figure 5, p=0,024, Kruskal-Wallis test).

Similarly to tBHQ, pretreatment with EtOH3% reduced inflammation in the lungs. Compared with controls, EtOH3% decreased the concentration of IL-1β (Figure 5E, p=0,002, Kruskal-Wallis test) and TNF-α (Figure 5F, p=0,031, Kruskal-Wallis test) in BALF and the phosphorylated p65/total p65 ratio (Figure 5, p=0,007, Kruskal-Wallis test) in lung tissue.

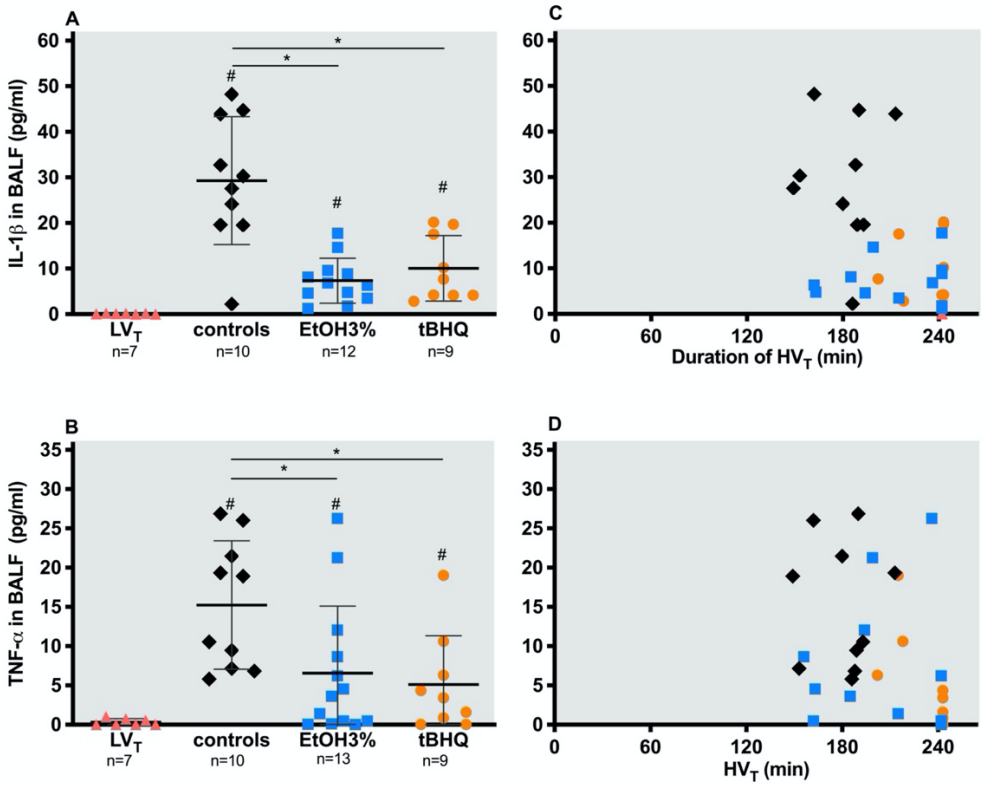

Figure 4. tBHQ: concentrations of IL-1β (A, C) and TNF-α (B, D) in BALF after HV_T ventilation. Mice were pretreated with PBS (controls, n=10), EtOH3% (n=12 in IL-1β and n=13 in TNF-α), or tBHQ (n=9), and then subjected to HV_T ventilation. A separate group of mice were pretreated with PBS and subjected to LV_T ventilation for 5 hours (LV_T, n=7). BALF was collected at the end of the experiment. *p< 0,05 tBHQ/EtOH3% versus controls (Kruskal-Wallis test). #p<0,05 LV_T versus HV_T (unpaired Student's t-test/Mann-Whitney U-test). Missing samples: 1 tBHQ (failure to collect BALF), 1 EtOH3% (not enough BALF). Two scatter plots were created to depict possible correlations between the concentrations of IL-1β (C) and TNF-α (D) in BALF and the duration of HV_T ventilation. No correlations were found for neither IL-1β (C) nor TNF-α (D) in any of the groups. Adapted from Veskemaa et. al. (83).

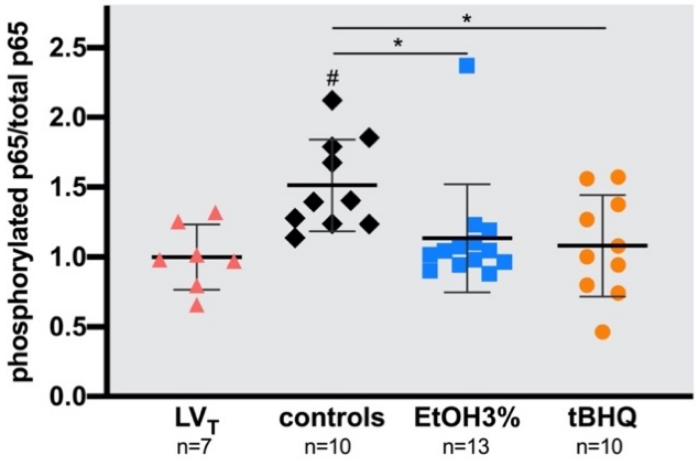

Figure 5. tBHQ: phosphorylated p65 and total p65 ratio in lung tissue after HV_T ventilation. Mice were pretreated with PBS (controls, n=10), EtOH3% (n=13), or tBHQ (n=10), and subjected to HV_T ventilation. A separate group of mice were pretreated with PBS and subjected to LV_T ventilation for 5 hours (LV_T, n=7). P65 was quantified with an ELISA in lung tissue collected at the end of the experiment. *p<0,05 tBHQ/EtOH3% versus controls. #p<0,05 LV_T versus HV_T. Adapted from Veskemaa et. al. (83).

6.6. tBHQ: pulmonary antioxidant Nrf2-dependent gene expression

To assess the antioxidant capacity of the lungs at baseline, we measured the Nrf2-dependent gene expression in the lungs of mice sacrificed one hour after pretreatment. Compared with controls at baseline (no HV_T), pretreatment with tBHQ yielded in a 1,7 fold increase of the relative expression of pulmonary messenger RNA (mRNA) of *Nrf2 (*Figure 6A, p<0,001, one-way ANOVA) and a 1,5 fold increase of *Sod1 (*Figure 6B, p=0,012, one-way ANOVA), but had no effect on the relative expression of *Gclc* (Figure 6C) and *Gclm* (Figure 6D). EtOH3% had no effect on the relative expression of these genes at baseline.

Compared with controls after HV_T ventilation, pretreatment with tBHQ resulted in a similar increase of Nrf2-dependent gene expression of *Nrf2* (Figure 6A), *Sod1* (Figure 6B) and *Gclc* (Figure 6C). Only the pulmonary gene expression of *Gclm* in mice subjected to HV_T ventilation were augmented by tBHQ (Figure 6D, p=0,014, Kruskal-Wallis test). EtOH3% had no effect on the relative expression of these genes after HV_T ventilation.

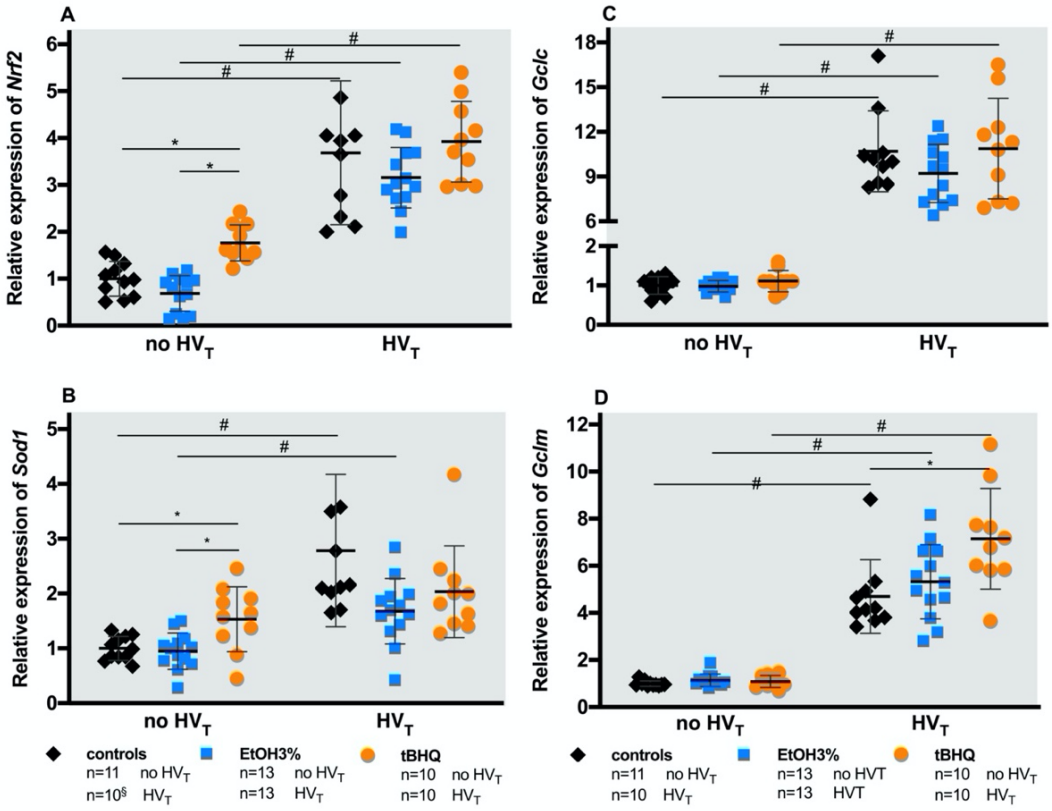

Figure 6. tBHQ: pulmonary messenger RNA (mRNA) concentrations of *Nrf2* (A), *Sod1* (B), *Gclc* (C) and *Gclm* (D). Mice were pretreated with PBS (controls), EtOH3%, or tBHQ, and either sacrificed one hour later (no HV_T) or subjected to HV_T ventilation. mRNA concentrations are expressed as fold change relative to the average expression values in no HV_T controls. *p<0,05 tBHQ versus controls/EtOH3% among no HV_T (One-way ANOVA), #p<0,05 no HV_T versus HV_T (unpaired Student's t-test/Mann-Whitney U-test). §1 outlier for *Nrf2* and 2 outliers for *Sod1* were included in the statistical analysis but are not depicted on this graph to increase visual comprehension. Adapted from Veskemaa et. al. (83).

6.7. tBHQ: pulmonary antioxidant proteins

EtOH3% and tBHQ had no effect on the total concentration of NRF2 protein (Figure 7A) and glutathione (Figure 7B) as well as GSH/GSSG ratio (Figure 8) at baseline. Compared with controls, pretreatment with tBHQ augmented the NRF2 protein concentration in lung tissue after HV_T ventilation (Figure 7A, 25 ± 3,2 pg/ml, p=0,030, one-way ANOVA). Pretreatment with tBHQ also provided the highest total pulmonary glutathione concentrations (Figure 7B, 48 ± 8 µmol/g tissue) compared with controls (Figure 7B, 36 ± 4 µmol/g tissue, p=0,004, one-way ANOVA) and EtOH3% (Figure 7B, 41 ± 6 µmol/g tissue, p=0,040, one-way ANOVA).

The GSH/GSSG ratio was similar among controls and mice pretreated with tBHQ or EtOH3% (Figure 8, HV_T).

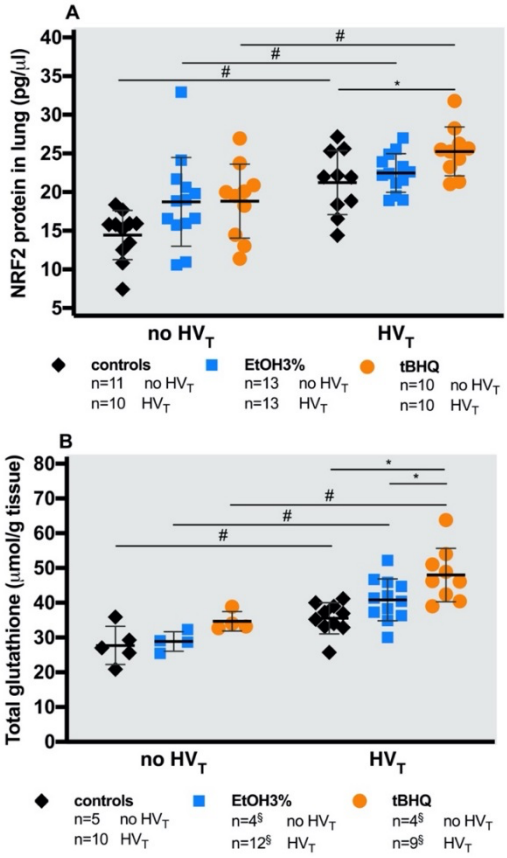

Figure 7. tBHQ: NRF2 protein (A) and total glutathione (B) concentration in lung tissue. Mice were pretreated with phosphate buffered saline (controls), EtOH3% or tBHQ, and either sacrificed one hour later (no HV_T) or subjected to HV_T ventilation. *p<0,05 tBHQ versus controls/EtOH3% among HV_T. #p<0,05 no HV_T versus HV_T. § 1 extreme outlier was excluded from analysis. Adapted from Veskemaa et. al. (83).

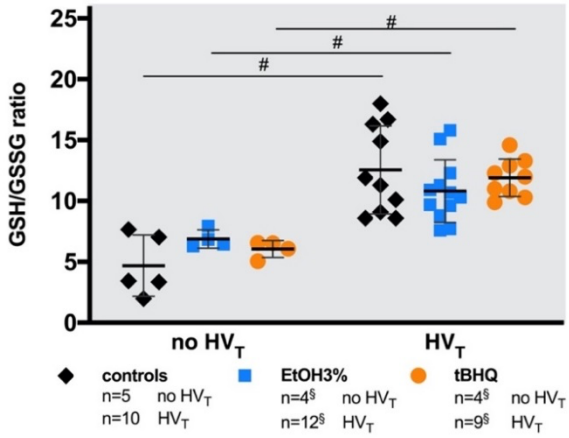

Figure 8. tBHQ: reduced glutathione (GSH) and oxidized glutathione (GSSG) ratio in lung tissue. Mice were pretreated with phosphate buffered saline (controls), EtOH3%, or tBHQ, and either sacrificed one hour later (no HV_T) or subjected to HV_T ventilation. #p<0,05 no HV_T versus HV_T. § 1 extreme outlier was excluded from analysis. Adapted from Veskemaa et. al.(83).

6.8. EGCG: survival and arterial blood oxygenation

Pretreatment with EGCG resulted in a survival of 20% of mice at 4 hours of HV_T ventilation, showing now survival benefit compared to the controls (Figure 9, p=0,0611, log-rank Mantel-Cox test). Compared to controls, EGCG did not improve arterial blood oxygenation (Figure 10, PaO_2 55 ± 21 mmHg, p=0,558, Mann-Whitney U-test).

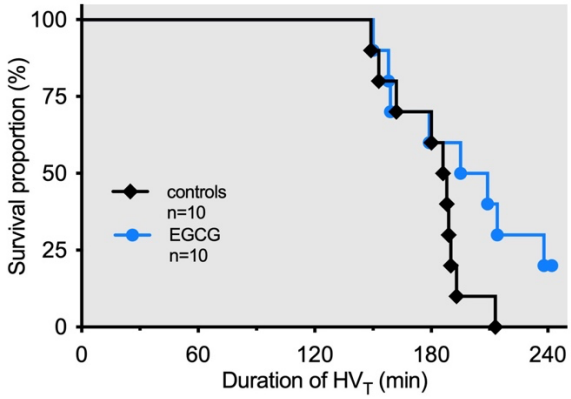

Figure 9. EGCG: survival of mice subjected to HV_T ventilation. Mice were pretreated with PBS (controls, n=10) or EGCG (n=10), and then subjected to HV_T ventilation. Mice were sacrificed when the mean arterial pressure decreased to less than 40 mmHg or after a maximum duration of 4 hours of HV_T ventilation.

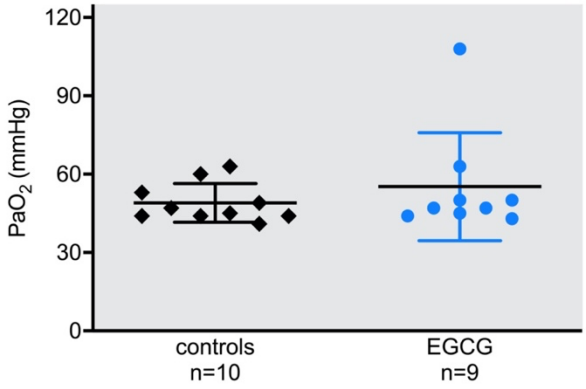

Figure 10. EGCG: arterial blood oxygen (PaO_2) tension of mice subjected to HV_T ventilation. Mice were pretreated with PBS (controls, n=10) or EGCG (n=10), and then subjected to HV_T ventilation. PaO_2 was measured in arterial blood obtained from the carotid artery at the end of the experiment. The inspired oxygen fraction was 0,4 in all groups. Missing samples: 1 in EGCG due failure to collect blood.

6.9. EGCG: lung edema and respiratory mechanics

Pretreatment with EGCG resulted in the same degree of lung edema compared to controls (Figure 11, 2,1 ± 0,5 µg/µl, p=0,651, unpaired Student's t-test). Compared to control, pretreatment with EGCG did not improve HV_T ventilation-associated reduction of dynamic (Table 3, 24 ± 3.7 µl/cm H_2O, p=0,739, Mann-Whitney U-test) or semistatic (Table 3, 30 ± 4,7 µl/cm H_2O, p=0,412, Mann-Whitney U-test) respiratory system compliance.

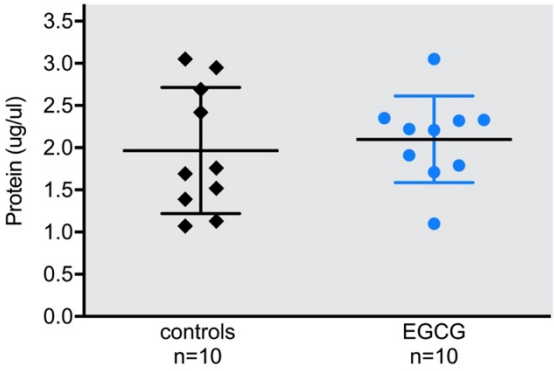

Figure 11. EGCG: total concentration of protein in BALF of mice subjected to HV_T ventilation. Mice were pretreated with PBS (controls, n=10) or EGCG (n=10), and then subjected to HV_T ventilation. As a surrogate for pulmonary edema, total protein concentration was measured in the broncho-alveolar lavage fluid (BALF) obtained after HV_T ventilation.

	Dynamic Respiratory System Compliance (µl/cm H_2O)		Semistatic Respiratory System Compliance (µl/cm H_2O))	
	Before HV_T	After HV_T	Before HV_T	After HV_T
controls	34 ± 1,7	22 ± 1,3$^{\#}$	57 ± 4,1	29 ± 2,7$^{\#}$
EGCG	35 ± 1,7	24 ± 3,7$^{\#}$	61 ± 2,8	30 ± 4,7$^{\#}$

Table 3. EGCG: respiratory system mechanics. Respiratory system mechanics measured before the initiation HV_T ventilation (before HV_T) and after HV_T ventilation (after HV_T) pretreated with PBS (controls, n≥ 9) or EGCG (n=10). All values presented mean±SD. #p<0.05 before HV_T versus after HV_T. Missing samples: 1 control for semistatic compliance (technical failure to record pressure-volume curves).

6.10. EGCG: inflammation in the lung

Compared to controls, pretreatment with EGCG resulted in a similar concentrations of pro-inflammatory cytokines in BALF: IL-6 (Figure 12A, p=0,265, unpaired Student's t-test), IL-1β (Figure 12B, p=0,218, Mann-Whitney U-test) and TNF-α (Figure 12C, p=0,137,unpaired Student's t-test).

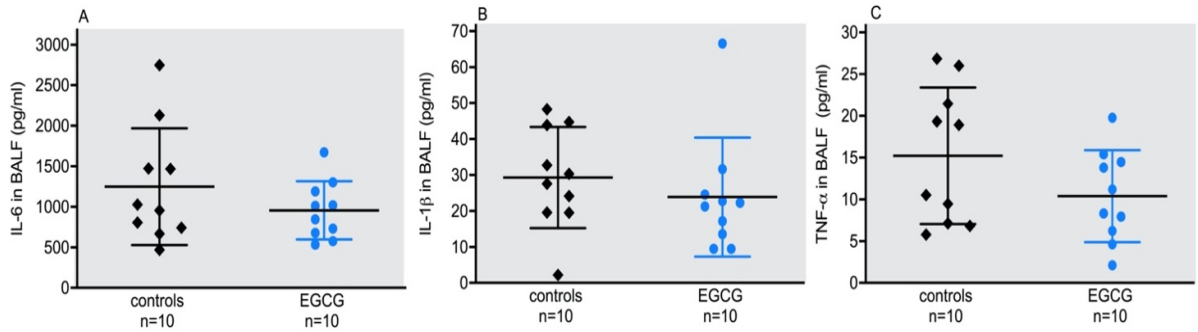

Figure 12. EGCG: concentrations of IL-6 (A), IL-1β (B) and TNF-α (C) in BALF after HV_Tventilation. Mice were pretreated with PBS (controls, n=10) or EGCG (n=10), and then subjected to HV_T ventilation. BALF was collected at the end of the experiment.

6.11. EGCG: pulmonary antioxidant Nrf2-dependent gene expression

Compared with controls pretreatment with EGCG increased the relative expression of *Nrf2* 1,4 fold at baseline (Figure 13A, p=0,065, unpaired Student's t-test). After HV_T ventilation pretreatment with EGCG yielded in a similar relative gene expression of Nrf2 as controls after HV_T ventilation(Figure 13A , p=0,396, unpaired Student's t-test). Pretreament with EGCG increased the relative expression of *Gpx2* 1,6 fold prior to mechanical ventilation (Figure 13B, p=0,004, Mann-Whitney U-test). After HV_T ventilation pretreatment with EGCG yielded in a 1.7 fold higher relative expression of *Gpx2* compared to controls (Figure 13B, p=0,011, unpaired Student's t-test).

EGCG also increased the expression of Nqo1 prior to mechanical ventilation 2,5 fold (Figure 13C, p=0,022, unpaired Student's t-test). After HV_T ventilation, pretreatment with EGCG yielded in a similar relative gene expression of Nqo1 (Figure 13C, p=0,529, Mann-Whitney U-test).

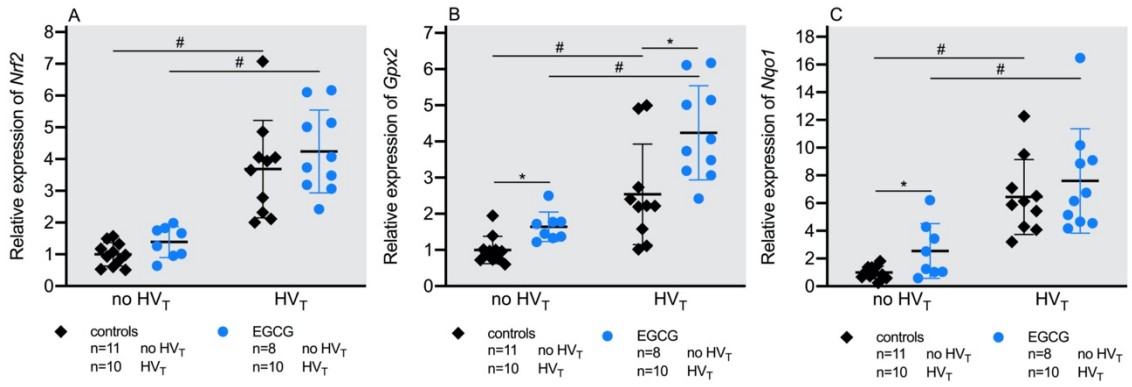

Figure 13. EGCG: pulmonary messenger RNA (mRNA) concentrations of *Nrf2* (A), *Gpx2* (B) and *Nqo1* (C). Mice were pretreated with PBS (controls) or EGCG, and either sacrificed one hour later (no HV_T) or subjected to HV_T ventilation. mRNA concentrations are expressed as fold change relative to the average expression values in no HV_T controls. *p<0,05 EGCG versus controls, #p<0,05 no HV_T versus HV_T

6.12. EGCG: pulmonary antioxidant proteins

Compared to controls pretreatment with EGCG did not increase total glutathione concentration (Figure 14A, p=0,352, unpaired Student's t-test) or reduce GSH/GSSG ratios (Figure 14B, p=0,354, unpaired Student's t-test) in the lung after HV_T ventilation.

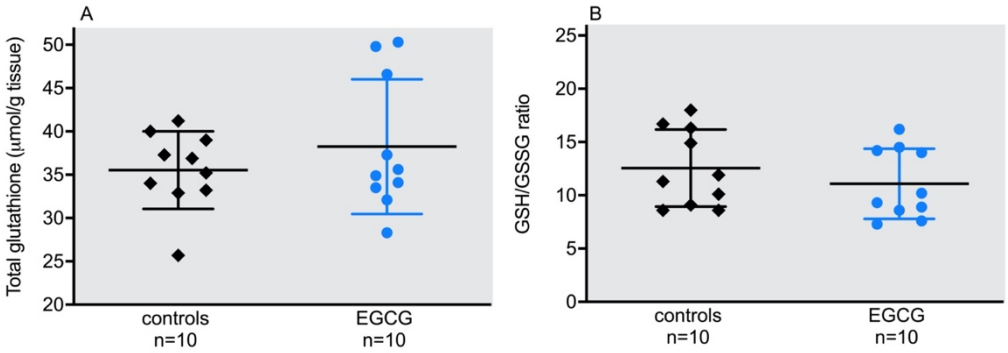

Figure 14. <u>EGCG:</u> total glutathione (A) concentration and GSH/GSSG ratio (B) after HV_T ventilation in lung tissue. Mice were pretreated with PBS (controls) or EGCG, and then subjected to HV_T ventilation. Lung tissue was collected at the end of experiment. Total glutathione (GSH+GSSG) and GSH/GSSG ratio was measured with a commercial colorimetric glutathione assay.

7. Discussion

In this mouse model of lethal VILI, pretreatment with tBHQ reduced the severity of acute lung injury and yielded in 60% survival rate. More specifically, pretreatment with tBHQ reduced deterioration of lung function (arterial blood oxygenation, respiratory system compliance) and development of pulmonary edema that was associated with the activation of the Nrf2-ARE pathway. In contrast, pretreatment with EGCG did not improve survival and neither reduced HV_T ventilation-associated deterioration of lung function nor development of pulmonary edema, albeit also activating the Nrf2-ARE pathway.

Activation of the Nrf2-ARE pathway during mechanical ventilation is undoubtedly an important protective mechanism against acute lung injury as Nrf2-deficient mice develop more severe VILI compared with wild type mice (55). Transcription factor Nrf2 plays a central role in this complex cytoprotective pathway that regulates glutathione metabolism, detoxification of ROS/RNS and xenobiotics, thioredoxin-dependent antioxidant system, NADPH regeneration as well as heme/iron metabolism (80). A single nucleotide polymorphism of Nrf2 has been shown to increase susceptibility to acute lung injury, characterized by hypoxemia, pulmonary inflammation and edema, in trauma patients (42). Furthermore, Nrf2-ARE pathway is interconnected with other signalling pathways regulating cell inflammation, cell proliferation and apoptosis (11). Therefore, Nrf2-deficiency reduces cellular ability to maintain homeostasis making cells more susceptible to any kind of damage.

The activation of the Nrf2-ARE is a complex process depending not only on the cellular abundancy and activity of Nrf2, but also on many co-factors as well as the cellular context. The latter meaning that under specific conditions only specific genes that are necessary to counteract a certain cellular insult are transcribed (80). Nrf2-dependent gene expression is also dependent on the inducing agent that activates the Nrf2-ARE pathway. Hence, this may explain why tBHQ and EGCG induced the expression of different genes. While tBHQ increased the relative gene expression of *Nrf2* and *Sod1* prior to mechanical ventilation (baseline), and *Gclm* after HV_T ventilation, EGCG increased the relative gene expression of *Nqo1* and *Gpx2* at baseline and *Gpx2* after HV_T ventilation. It is possible that the different Nrf2-dependent gene expression profile explains the different functional outcome.

Firstly, pretreatment with tBHQ increased the pulmonary relative gene expression of *Nrf2* prior to mechanical ventilation resulting in an increased NRF2 protein concentration after HV_T ventilation. It is known that NRF2 autoregulates its own transcription as ARE is also found at the promoter region of *Nrf2*. This positive feedback amplifies the activation of the Nrf2-ARE pathway creating an abundance of NRF2 in the cells that might provide additional cytoprotection in the presence of oxidative stress (11). *Nrf2* promoter region also contains a binding site for NF-kB which induces the transcription of *Nrf2* during inflammation. In our study, *Nrf2* transcription was increased at baseline, when the level of inflammation was low, showing that tBHQ induced the transcription of *Nrf2*. Interestingly, NRF2 protein concentration was increased after HV_T ventilation but not at baseline (one hour after pretreatment), although tBHQ is known to increase the stability of NRF2 by modifying Keap1 cysteine residues that inhibit ubiquitylation and subsequent proteosomal degradation of NRF2 (75). It is possible that NRF2 protein concentration was increased at some timepoint after pretreatment, but it had already been degraded within one hour after tBHQ injection, therefore only increased *Nrf2* expression was measurable at baseline. However, the increased NRF2 protein concentration after HV_T ventilation could reflect the longer time required for protein synthesis. In addition, the massively increased relative gene expression of *Nrf2* after HV_T ventilation in all groups possibly overrode the activation of the Nrf2-ARE pathway attributable to tBHQ.

Secondly, pretreatment with tBHQ increased the relative expression of *Sod1* at baseline. *Sod1* encodes Cu/Zn superoxide dismutase (Cu/Zn SOD) or otherwise known superoxide dismutase one that catalyses the chemical reaction converting superoxide radical into oxygen and hydrogen peroxide. Subsequently, hydrogen peroxide that is also harmful is metabolized either into water and oxygen by an enzyme called catalase in the peroxisomes or into water and glutathione by glutathione peroxidase disulfide in the mitochondria and cytosol. Thus, the overall result is the reduction of oxidative stress. There is some evidence, that Cu/Zn SOD plays an important role in the protection against VILI. Intravenous administration of Cu/Zn SOD during HV_T ventilation prevents lung function impairment and lung injury via reducing pulmonary oxidative stress and lung inflammation in rats (69, 86). In addition, a study demonstrated that administration of human IL-10 to rats can restore the HV_T ventilation related reduction of SOD activity and provides protection against VILI (16). Moreover,

increased activity of SOD was associated with the protective effect of chloroquine in a mouse model using paraquat, a common herbicide, to induce acute lung injury (65).

Thirdly, pretreatment with tBHQ increased the relative gene expression of *Gclm* after HV_T ventilation but not at baseline. *Gclm* encodes the glutamate-cysteine ligase modifier subunit (GCLM). Glutamate-cystein ligase (GCL) is the first rate limiting enzyme in the glutathione biosynthesis. GCLM makes GCL catalytically more efficient and less prone to allosteric inhibition by the cellular concentration of glutathione, thus inhibiting negative feedback (41). At baseline, when there was no oxidative stress, the relative expression of *Gclm* was not increased, likely because glutathione biosynthesis was not necessary at that point. This reflects dependency of Nrf2-dependent gene on the cellular context. Under oxidative stress, during HV_T ventilation, when glutathione was used to maintain redox balance, the gene expression of *Gclm* was initiated. Hence, the production of glutathione was increased and therefore pretreatment with tBHQ resulted in the highest total glutathione concentration after HV_T ventilation. Glutathione is a major antioxidant that protects cells against oxidative injury. Glutathione peroxidases and glutathione-S-transferases use glutathione as a substrate to reduce organic peroxides and to detoxify xenobiotics, respectively. Therefore, glutathione metabolism plays a central role in cytoprotection. Reduced synthesis of glutathione is part of the aging process and is involved in the development in many chronic pulmonary diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, and cystic fibrosis (32). Abundance of glutathione has shown to provide protection against VILI. N-acetylcysteine that provides essential cysteine for glutathione biosynthesis has shown to attenuate VILI in isolated rat lungs (18). Furthermore, higher total glutathione concentration was associated with the protective effect of sodium sulphide in a mouse model of VILI (24).

Interestingly, up-regulation of the relative gene expression of *Gpx2* and *Nqo1* did not provide protection against VILI, although Nrf2-dependent induction of these genes has been previously associated with protection against lung injury (76). *Gpx2* encodes glutathione peroxidase 2 (GPX2), an enzyme that uses glutathione as a substrate to reduce hydrogen peroxide to water and organic peroxides to respective alcohols. Although, induction of *Gpx2* has been shown to protect against cigarette smoking-induced oxidative stress in the lung, it did not provide protection against VILI (66). One reason could be that the severity of oxidative stress caused by lethal HV_T ventilation

exceeded the possible protection provided by *Gpx2*. In addition, our data show that EGCG did not increase the total concentration of glutathione. Lack of substrate might have limited the protective effect of GPX2. Furthermore, the concentration of GPX2 in the lung was not measured. Therefore, it remains unclear, whether in this case increased gene expression correlates with increased protein levels. *Nqo1* encodes NAD(P)H dehydrogenase [quinone] 1 (NQO1) that reduces quinones to hydroquinones thus preventing the production of ROS/RNS. There is contradictory evidence about the protective effect of NQO1 in acute lung injury. Genetic polymorphism that reduces the transcription of NQO1 has shown to be protective against acute lung injury in patients with major trauma (60). However, lack of NQO1 has shown to increase susceptibility to hyperoxic acute lung injury in mice (20). In our model, the mice were ventilated with 40% of oxygen that is not considered dangerous (38).

Another difference at the biochemical level was that in contrast to EGCG, tBHQ decreased the level of pro-inflammatory cytokines. The reduction of pro-inflammatory response to HV_T ventilation was associated with reduced activation of the NF-κB canonical pathway indicated by the reduced phosphorylated p65/total p65 ratio. Similarly, EtOH3% that was used as a vehicle for poorly water soluble tBHQ also reduced the pro-inflammatory response to HV_T ventilation and was associated with the activation of the canonical pathway of NF-κB. It is known that there is a cross-talk between the Nrf2-ARE pathway and NF-κB pathway. Moreover, there is evidence that tBHQ can activate Nrf2-mediated inhibition of the p65 NF-κB pathway (85). One the other hand, there is also evidence that ethanol has anti-inflammatory effects in human lung cells. This anti-inflammatory effect of ethanol was associated with the inhibition of p50 in the canonical pathway of NF-κB (50). P50 forms a heterodimer with p65 that acts like a transcription factor involved in the expression pro-inflammatory genes (40). Furthermore, p65 has shown to interact with Keap1 directly and inhibit the activation of the Nrf2-ARE pathway (88). In our study, albeit pretreatment with EtOH3% reduced phosphorylated p65/total p65 ratio, it did not have any effect on the activation of the Nrf2-ARE pathway. Overall, it is fair to assume that in our study EtOH3% probably had a synergistic effect with tBHQ.

In contrary to many other authors, in our study, pretreatment with EGCG did not reduce the levels of pro-inflammatory cytokines in BALF. In a paraquat-induced acute lung injury model, 5mg/kg EGCG reduced the levels of IL-6, IL-1β and TNF-α in BALF (64).

A striking difference to our mouse model was that the mice were sacrificed 3 days after the injections, indicating that the paraquat-induced lung injury was not lethal, thus being less severe. Similarly, in an LPS-induced acute lung injury model, mice pretreated with 10mg/kg EGCG one hour before intratracheal administration of LPS showed reduction of inflammation, including lower levels of TNF-α in BALF (6). In that model the mice were sacrificed 24 hours later meaning that the lung injury was not lethal. In addition, pretreatment with 40 mg/kg EGCG provided protection against fluoride-induced oxidative stress mediated lung injury in rats (63). In that model, EGCG was administered chronically for 4 weeks and a much higher dosage was used. In our study, dose response experiments showed no extra benefits in using higher dosages than 5mg/kg of EGCG. Furthermore, it is well known that EGCG is hepatotoxic and its maximal tolerable dosage for chronic use is 45mg/kg in mice (84). Overall, differences in timing and dosage as well as differences between the diverse pathogenetic models of acute lung injury might explain the different effect of EGCG in each specific animal model.

7.1. Limitations

The protective effect of pretreatment with tBHQ in this mouse model of VILI is merely associated with the activation of the Nrf2-ARE pathway. In order to confirm whether protection against acute lung injury in this mouse model of VILI is Nrf2 mediated, experiments that involve blocking the Nrf2-ARE pathway (knock-out mice, small interfering RNA) need to be performed.

The use of very severe VILI model that was lethal to all the control animals might have limited the possible positive effect that activation of the Nrf-ARE pathway could have had during less injurious mechanical ventilation. It is fair to assume that in this mouse model of VILI, where tidal volumes exceeded the total lung capacity, mechanical ventilation not only caused biochemical injury, but also severe mechanical injury. VILI models using smaller tidal volumes could provide more insight whether EGCG can provide any protection.

The activation of the Nrf2-ARE was measured mostly at the gene level, especially concerning the results of EGCG. It would be of interest to know whether increased gene expression translated into higher protein concentrations.

7.2. Summary

Specific activation of the Nrf2-ARE pathway with tBHQ provided protection against VILI in this mouse model. On the other hand, specific activation of Nrf2-ARE pathway with EGCG did not provide any protection against acute lung injury in this mouse model of VILI. The activation of the Nrf2-ARE pathway by tBHQ and EGCG resulted in a distinct Nrf2-dependent gene expression profile that might explain why tBHQ provided protection against VILI but EGCG did not. Additionally, an anti-inflammatory effect of EtOH3% mediated by NF-κB suppression might have contributed to the protective effect of tBHQ during mechanical ventilation.

This study confirms, that specific activation of the Nrf2-ARE pathway poses an attractive drug target to prevent VILI. Further studies are necessary to test the effectiveness of tBHQ in larger animal models of VILI. Moreover, it would be interesting to test other Nrf2 inducers that might have an even more favourable Nrf2-ARE pathway activation profile.

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9. Statutory Declaration

"I, Lilly Veskemaa, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic "Pharmacological activation of the Nrf2-ARE pathway and its protective effects in an experimental model of ventilator-induced lung injury/ Protektiver Effekt einer pharmakologischen Aktivierung des Nrf2-ARE Signalweges in einem experimentellen Modell des beatmungs-induzierten Lungenschadens , independently and without the support of third parties, and that I used no other sources and aids than those stated.
All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me."

Date Signature

9.1. Declaration of your own contribution to the top-journal publication for a PhD or MD/PhD degree

Veskemaa L, Graw JA, Pickerodt PA, Taher M, Boemke W, Gonzalez-Lopez A, and Francis RCE. Tert-butylhydroquinone augments Nrf2-dependent resilience against oxidative stress and improves survival of ventilator-induced lung injury in mice. *Am J Physiol Lung Cell Mol Physiol* 2020.

Contributions of Lilly Veskemaa:

- Setup, refinement and calibration of the mouse model of VILI
- Experimental procedures for all dose-response definitions of drugs, including i.p./i.v. injections, harvesting of organs, biochemical tests and data analysis.
- Planning and execution of all experimental procedures involving mechanical ventilation
- Planning and execution of the vast majority of laboratory bench work, except few additional biochemical measurements required in the course of the revision process before final acceptance of the manuscript for publication
- All statistical analyses
- Contribution to the discussion and interpretation of data
- Design and completion of all graphs and tables
- Drafting of the manuscript and inclusion of revisions from co-authors, including editorial revision before final acceptance for publication

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Signature, date and stamp of first supervising university professor / lecturer

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Signature of doctoral candidate

10. Extract from the Journal Summary List

Journal Data Filtered By: **Selected JCR Year: 2018** Selected Editions: SCIE,SSCI
Selected Categories: **"PHYSIOLOGY"** Selected Category Scheme: WoS
Gesamtanzahl: 81 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	PHYSIOLOGICAL REVIEWS	28,672	24.250	0.026830
2	Annual Review of Physiology	9,562	17.902	0.012810
3	JOURNAL OF PINEAL RESEARCH	10,695	15.221	0.010560
4	PHYSIOLOGY	3,499	6.380	0.005880
5	Comprehensive Physiology	4,044	6.246	0.010750
6	Reviews of Physiology Biochemistry and Pharmacology	738	6.214	0.000540
7	International Journal of Behavioral Nutrition and Physical Activity	9,914	6.037	0.020780
8	Acta Physiologica	4,734	5.868	0.008300
9	JOURNAL OF PHYSIOLOGY-LONDON	52,037	4.950	0.041100
10	EXERCISE AND SPORT SCIENCES REVIEWS	3,157	4.739	0.003010
11	JOURNAL OF CELLULAR PHYSIOLOGY	20,046	4.522	0.018580
12	JOURNAL OF GENERAL PHYSIOLOGY	8,352	4.258	0.008260
13	AMERICAN JOURNAL OF PHYSIOLOGY-ENDOCRINOLOGY AND METABOLISM	19,036	4.125	0.017240
14	AMERICAN JOURNAL OF PHYSIOLOGY-LUNG CELLULAR AND MOLECULAR PHYSIOLOGY	13,709	4.060	0.016530
15	AMERICAN JOURNAL OF PHYSIOLOGY-HEART AND CIRCULATORY PHYSIOLOGY	27,828	4.048	0.022820
16	International Journal of Sports Physiology and Performance	4,936	3.979	0.009660
17	AMERICAN JOURNAL OF PHYSIOLOGY-GASTROINTESTINAL AND LIVER PHYSIOLOGY	14,295	3.729	0.014090

11. Publication

Veskemaa L, Graw JA, Pickerodt PA, Taher M, Boemke W, Gonzalez-Lopez A, and Francis RCE. Tert-butylhydroquinone augments Nrf2-dependent resilience against oxidative stress and improves survival of ventilator-induced lung injury in mice. *Am J Physiol Lung Cell Mol Physiol* 2020.

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RESEARCH ARTICLE

Tert-butylhydroquinone augments Nrf2-dependent resilience against oxidative stress and improves survival of ventilator-induced lung injury in mice

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Abstract

Oxidative stress caused by mechanical ventilation contributes to the pathophysiology of ventilator-induced lung injury (VILI). A key mechanism maintaining redox balance is the upregulation of nuclear factor-erythroid-2-related factor 2 (Nrf2)-dependent antioxidant gene expression. We tested whether pretreatment with an Nrf2-antioxidant response element (ARE) pathway activator *tert*-butylhydroquinone (tBHQ) protects against VILI. Male C57BL/6J mice were pretreated with an intraperitoneal injection of tBHQ (n = 10), an equivalent volume of 3% ethanol (EtOH3%, vehicle, n = 13), or phosphate-buffered saline (controls, n = 10) and were then subjected to high tidal volume (HV_T) ventilation for a maximum of 4 h. HV_T ventilation severely impaired arterial oxygenation (Pa_{O_2} = 49 ± 7 mmHg, means ± SD) and respiratory system compliance, resulting in a 100% mortality among controls. Compared with controls, tBHQ improved arterial oxygenation (Pa_{O_2} = 90 ± 41 mmHg) and respiratory system compliance after HV_T ventilation. In addition, tBHQ attenuated the HV_T ventilation-induced development of lung edema and proinflammatory response, evidenced by lower concentrations of protein and proinflammatory cytokines (IL-1β and TNF-α) in the bronchoalveolar lavage fluid, respectively. Moreover, tBHQ enhanced the pulmonary redox capacity, indicated by enhanced Nrf2-depentent gene expression at baseline and by the highest total glutathione concentration after HV_T ventilation among all groups. Overall, tBHQ pretreatment resulted in 60% survival ($P < 0.001$ vs. controls). Interestingly, compared with controls, EtOH3% reduced the proinflammatory response to HV_T ventilation in the lung, resulting in 38.5% survival (P = 0.0054 vs. controls). In this murine model of VILI, tBHQ increases the pulmonary redox capacity by activating the Nrf2-ARE pathway and protects against VILI. These findings support the efficacy of pharmacological Nrf2-ARE pathway activation to increase resilience against oxidative stress during injurious mechanical ventilation.

lung; redox; tBHQ; ventilation; VILI

INTRODUCTION

Every year ~800,000 critically ill patients require mechanical ventilation in the United States (42). Although often life-saving, mechanical ventilation can induce or aggravate lung injury by causing ventilator-induced lung injury (VILI) (14). VILI is a serious complication of mechanical ventilation that can lead to multiple-organ failure and death (10). Protective ventilation strategies such as the use of low tidal volumes and techniques to improve respiratory mechanics such as prone positioning reduce mortality among patients with the adult respiratory distress syndrome (ARDS), attributed at least partly to the reduction of VILI (5, 16). The pathophysiology of VILI involves mechanical injury from volu-, baro-, and atelectrauma as well as biochemical injury from inflammation and oxidative stress (39). Cyclic stretching of the epithelial cells lining the alveoli leads to the release of various mediators including proinflammatory cytokines and reactive oxygen/nitrogen species (ROS/RNS), causing inflammation and oxidative stress in the lung (7, 11, 30). Cyclic stretching also affects the pulmonary endothelium. High tidal volume ventilation activates stretch-induced cation-channel transient receptor potential vanilloid-4 (TRPV-4), leading to inflammation and vascular hyperpermeability (27). In addition, activation of TRPV-4 in alveolar macrophages induces the production of ROS/RNS (18) and thus contributes to oxidative stress. Local biochemical injury can progress systemically and damage extrapulmonary organs, contributing to multiple-organ failure and death of critically ill patients (10).

An adaptive response to oxidative stress is the increased expression of antioxidant genes. Many of these genes are regulated by nuclear factor-erythroid-2-related factor 2 (Nrf2), a master regulator of antioxidant gene expression. In response to oxidative stress, Nrf2 dissociates from its

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cytoplasmatic inhibitor Keap1, translocates to the nucleus, and binds to the antioxidant response element (ARE) in the upstream promoter region of many antioxidant genes, where it will initiate their transcription (6). These genes include *Nrf2* per se and many other antioxidant genes (e.g., *Sod1*, *Gpx2*, and *Nqo1*) encoding antioxidant and phase II detoxification proteins/enzymes that play a pivotal role in maintaining the cellular redox status. Oxidative stress, therefore, is a physiological activator of the Nrf2-ARE pathway.

Pharmacological activation of the Nrf2-ARE pathway can be induced by Nrf2-ARE pathway activators such as *tert*-butylhydroquinone (tBHQ). By modifying the cysteine residues of Keap1, tBHQ enables the translocation of Nrf2 to the nucleus (1, 37). It has been shown that tBHQ protected the kidney and liver by attenuating oxidative stress in rat models of ischemia-reperfusion injury (17, 35). In addition, pharmacological activation of the Nrf2-ARE pathway with tBHQ reduced mortality and severity of lung damage in mice with LPS-induced lung injury (41). In contrast, in an *Nrf2*-knockout model, genetic deficiency of Nrf2 aggravated the severity of lung injury from mechanical ventilation in mice (31). Therefore, Nrf2 seems to play a pivotal role in the protection of organs against biochemical and mechanical insults associated with inflammation and oxidative stress.

Based on these considerations, we hypothesized that activation of the Nrf2-ARE pathway with tBHQ before mechanical ventilation would increase the antioxidant response during mechanical ventilation and thus protect against the detrimental effects of mechanical ventilation. Therefore, in this study, we investigate whether activation of the Nrf2-ARE pathway by pretreatment with tBHQ attenuates the development of VILI triggered by high tidal volume mechanical (HV_T) ventilation in mice.

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee (Tierversuchskommission, Landesamt für Gesundheit und Soziales, Berlin, Germany, Approval number G0189/15). Male 8- to 10-wk old C57BL/6J mice were obtained from Charles River Laboratories and kept under standard laboratory conditions provided by the Animal Facility of Charité – Universitätsmedizin Berlin (FEM Forschungseinrichtung für Experimentelle Medizin, Tierhaltung).

Mouse model of VILI.

Mice (weight, 24.7 ± 0.8 g) were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (6 mg/kg). A surgical tracheostomy was performed, and mice were ventilated in a volume-controlled mode at a tidal volume (V_T) of 10 mL/kg, respiratory rate of 90 breaths/min, positive end-expiratory pressure (PEEP) of 2 cmH_2O, and a fraction of inspired oxygen (F_IO_2) of 0.4 for 1 h (baseline ventilation). The common carotid artery was catheterized for blood pressure monitoring, continuous infusion of maintenance anesthesia, and blood sampling. One hour after the initiation of baseline ventilation, high tidal volume (HVT) ventilation was started—V_T was increased to 40–42.5 mL/kg to reach a peak inspiratory airway pressure (PIP) of 30 cmH_2O. The PEEP was lowered to 1 cmH_2O to induce high-stretch mechanical ventilation with enough stress and strain to cause mortality within 4 h of ventilation. In addition, the respiratory rate was reduced to 60 breaths/min, and 3.5% of CO_2 was added to the inhaled gas (40% oxygen concentration) to prevent respiratory alkalosis. Mice were ventilated for a maximum duration of 4 h of HV_T ventilation or until the mean arterial pressure dropped below 40 mmHg for more than 10 s. Alveolar recruitment maneuvers were performed every 30 min during baseline ventilation and every 60 min during HV_T ventilation. Body temperature was maintained around 37.5°C with a thermostatically controlled heating pad.

Experimental groups.

Male C57BL/6J mice were randomly assigned to receive a single intraperitoneal injection of *tert*-butylhydroquinone (tBHQ, Sigma-Aldrich; 5 mg/kg, 8 mL/kg, n = 10), 3% ethanol (EtOH3%, 8 mL/kg, n = 13), or Dulbecco's phosphate-buffered saline (DPBS, Gibco, Thermo Fisher Scientific, 8 mL/kg, n = 10) 1 h before anesthesia induction.

Tert-BHQ was dissolved in 96% ethanol (Dr. K. Hollborn & Söhne GmbH & Co. KG, Germany) and then diluted with ultra-pure water, to yield a final concentration of 620 mg/L in 3% ethanol. The pH was adjusted to 7.4 with 0.1M NaOH.

Dose-response experiments were performed to determine the optimal dose of tBHQ yielding a significant increase in Nrf2-dependent antioxidant gene expression (Fig. 1). To this end, mice received a single intraperitoneal injection of tBHQ (5, 10, or 50 mg/kg), or equivalent volumes of EtOH3%, or DPBS, 1 h before being anesthetized and euthanized to analyze Nrf2-dependent antioxidant gene expressions in the lung tissue. In addition, these mice were used as nonventilated controls to determine the baseline physiological status after injection of tBHQ, EtOH3%, or DPBS without the effect of HV_T ventilation.

Another group of mice were pretreated with DPBS (8 mL/kg) and subjected to low tidal volume ventilation (LV_T, 10 mL/kg). These mice were handled identical to HV_T groups, with the only difference being that the V_T was kept at the baseline level (10 mL/kg) for 5 h.

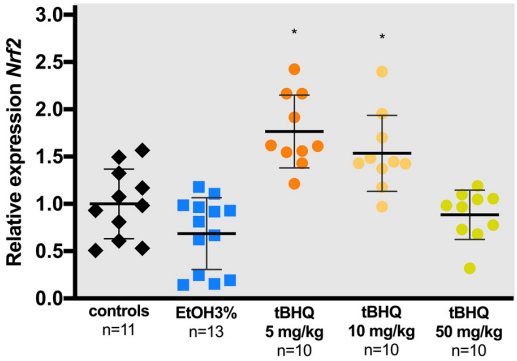

Figure 1. Best dosage selection of tBHQ. Mice received a single intraperitoneal injection of tBHQ (5, 10, or 50 mg/kg), or equivalent volumes of 3% ethanol (EtOH3%), or phosphate-buffered saline (controls), 1 h before being anesthetized and euthanized. The relative expression of *Nrf2* was measured in the lung tissue. *P < 0.05 tBHQ 5 mg/kg vs. controls/EtOH3%/tBHQ 50 mg/kg; tBHQ 10 mg/kg vs. controls/EtOH3%/tBHQ 50 mg/kg (one-way ANOVA). tBHQ, *tert*-butylhydroquinone.

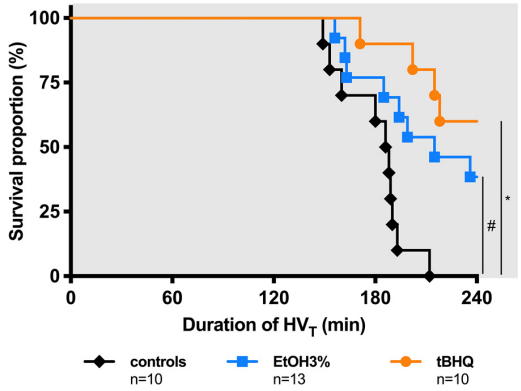

Figure 2. Survival of mice subjected to high tidal volume (HV_T) ventilation. Mice were pretreated with phosphate-buffered saline (controls), 3% ethanol (EtOH3%), or *tert*-butylhydroquinone (tBHQ) and subjected to HV_T ventilation. Mice were euthanized when the mean arterial pressure decreased to less than 40 mmHg or after a maximum duration of 4 h of HV_T ventilation. **P* = 0.0001 tBHQ vs. controls, #*P* = 0.0054 EtOH3% vs. controls (log-rank Mantel–Cox test).

Evaluation of respiratory system mechanics and blood gas analysis.

We measured V_T, peak inspiratory airway pressure (PIP), positive end-expiratory pressure (PEEP), inspiratory capacity, compliance of the respiratory system, pressure-volume curves, and partial pressures of oxygen in arterial blood, as described with more details in the Supplemental material (all Supplemental material available at https://doi.org/10.6084/m9.figshare.12895790).

Evaluation of pulmonary edema and inflammation.

The total protein concentration in the bronchoalveolar lavage fluid (BALF) was measured with the bicinchoninic acid assay (Pierce BCA Protein Assay, Thermo Fisher Scientific) to assess pulmonary edema formation.

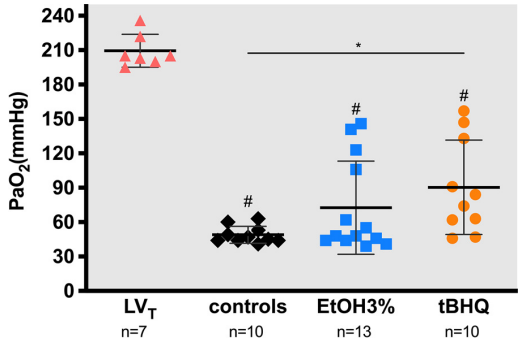

Figure 3. Arterial blood oxygen (Pa_{O_2}) tension of mice subjected to high tidal volume (HV_T) ventilation. Mice were pretreated with phosphate-buffered saline (controls, *n* = 10), 3% ethanol (EtOH3%, *n* = 13), or *tert*-butylhydroquinone (tBHQ, *n* = 10), and then subjected to HV_T ventilation. A separate group of mice were pretreated with phosphate-buffered saline and subjected to low tidal volume ventilation for 5 h (LV_T, *n* = 7). Pa_{O_2} was measured in arterial blood obtained from the carotid artery at the end of the experiment. The inspired oxygen fraction was 0.4 in all groups. **P* = 0.0181 tBHQ vs. controls (Kruskal–Wallis test). #*P* < 0.05 LV_T vs. HV_T (unpaired Student's *t* test).

The concentrations of proinflammatory cytokines interleukin-6 (IL-6), interleukin-1-β (IL-1β), and tumor necrosis factor-α (TNF-α) were measured using uncoated ELISA kits (Invitrogen by Thermo Fisher Scientific). Phosphorylated p65/total p65 ratios were determined using the NF-κB p65 (pS536 + total) ELISA kit (Abcam, UK).

Evaluation of oxidative stress and pulmonary gene expression.

Reduced (GSH), oxidized (GSSG), and total (GSH + GSSG) glutathione concentrations in lung tissue were measured using an enzymatic assay (Cayman Chemical).

Quantitative real-time polymerase chain reaction was used to determine the relative gene expression of pulmonary nuclear factor (erythroid-derived 2)-like 2 (*Nrf2*), pulmonary superoxide dismutase 1 (*Sod1*), and glutamate-cysteine ligase

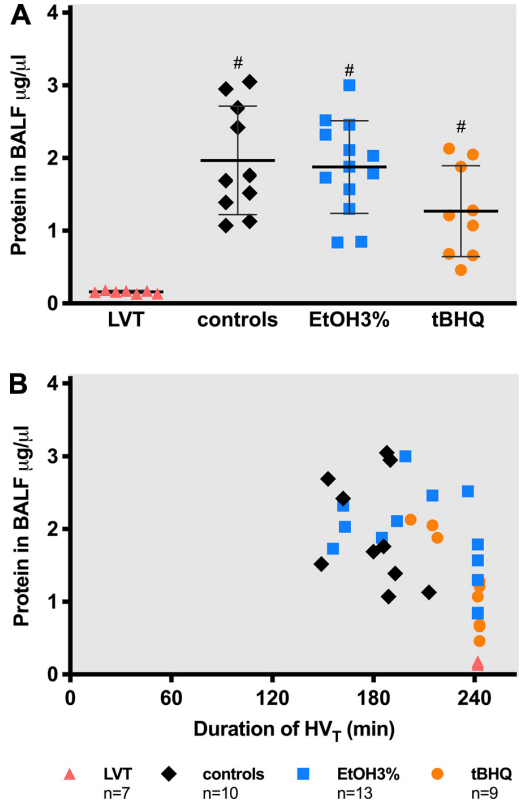

Figure 4. Total concentration of protein in BALF of mice subjected to high tidal volume (HV_T) ventilation. Mice were pretreated with phosphate-buffered saline (controls, *n* = 9), 3% ethanol (EtOH3%, *n* = 13), or *tert*-butylhydroquinone (tBHQ, *n* = 10) and then subjected to HV_T ventilation. A separate group of mice were pretreated with phosphate-buffered saline and subjected to low tidal volume ventilation for 5 h (LV_T, *n* = 7). As a surrogate for pulmonary edema, total protein concentration was measured in the bronchoalveolar lavage fluid (BALF) obtained after LV_T and HV_T ventilation (*A*). #*P* < 0.05 LV_T vs. HV_T (unpaired Student's *t* test). Missing samples: 1 tBHQ; failure to collect BALF. A scatter plot (*B*) was created to depict possible correlations between the total concentration of protein in BALF and the duration of HV_T ventilation. Only in the tBHQ subgroup, a strong negative correlation ($r = -0.892$, $P = 0.001$, Pearson correlation) was found.

catalytic (*Gclc*) and modifier (*Gclm*) subunits. Total concentrations of NRF2 protein in lung tissue were measured using an NRF2 (NF-E2-related factor 2) ELISA kit (Fine Test, China).

Further detailed description of the biochemical and molecular biological procedures is available in the Supplemental material.

Statistical analysis.

The Shapiro–Wilk test was used to assess data distribution. Normally distributed data were assessed with unpaired Student's *t* test (comparison of two groups) or one-way ANOVA with Bonferroni correction for multiple testing (comparison of >two groups). For comparison of groups with nonparametric data, the Mann–Whitney *U* test (comparison of two groups) or the Kruskal–Wallis test with Dunn's correction for multiple testing (comparison of more than two groups) was used. Compliance of the respiratory system before and after HV_T ventilation was compared with a two-way ANOVA for repeated measures and Bonferroni correction for multiple testing. Survival curves were compared with the log-rank Mantel–Cox test. Correlations were calculated using the Pearson correlation coefficient or nonparametric Spearman correlation.

All tests were performed with GraphPad Prism v5.01 for Windows (GraphPad Software, San Diego, CA). Data are expressed as means ± standard deviation (SD). A P value less than 0.05 was considered statistically significant. The sample size was based on our previous experience with this design.

RESULTS

tBHQ improves survival and arterial oxygenation in mice subjected to HV_T ventilation.

HV_T ventilation caused 100% mortality within 4 h among controls (Fig. 2). Pretreatment with tBHQ and EtOH3% significantly improved survival: 60% and 38.5% of mice were alive at 4 h of HV_T ventilation, respectively (Fig. 2).

Mice subjected to LV_T ventilation invariably survived mechanical ventilation and had a normal arterial oxygenation (Pa_{O_2} of 209 ± 14 mmHg, F_IO_2 of 0.4) at the end of the experiment (Fig. 3). HV_T ventilation severely impaired arterial oxygenation in all groups. Compared with controls, tBHQ improved arterial oxygenation at the end of HV_T ventilation (Pa_{O_2} of 90 ± 41 mmHg vs. 49 ± 7 mmHg, P = 0.018) (Fig. 3). EtOH3% did not improve arterial oxygenation at the end of HV_T ventilation compared with controls (Pa_{O_2} of 73 ± 42 mmHg, P = 0.813) (Fig. 3).

Table 1. *Respiratory system mechanics*

	Dynamic Respiratory System Compliance, μL/cmH_2O		Semistatic Respiratory System Compliance, μL/cmH_2O	
	Before HV_T	After HV_T	Before HV_T	After HV_T
Controls	34 ± 1.7	22 ± 1.3$^{\#}$	57 ± 4.1	29 ± 2.7$^{\#}$
EtOH3%	35 ± 1.8	26 ± 4.4$^{\#}$	60 ± 3.3	32 ± 6.1$^{\#}$
tBHQ	35 ± 1.5	28 ± 5.1$^{*\#}$	60 ± 3.0	37 ± 9.0$^{*\#}$

Values are means ± SD. Respiratory system mechanics measured before the initiation high tidal volume ventilation (before HV_T) and after HV_T ventilation (after HV_T) pretreated with phosphate-buffered saline (controls, $n \geq 9$), 3% ethanol (EtOH3%, n = 13), or *tert*-butylhydroquinone (tBHQ, $n \geq 9$). $^*P < 0.05$; tBHQ vs. controls after HV_T (one-way ANOVA). $\#P < 0.05$; before HV_T vs. after HV_T (two-way repeated-measures ANOVA). Missing samples: 1 control and 1 tBHQ in semistatic compliance due to technical failure to record pressure-volume curves.

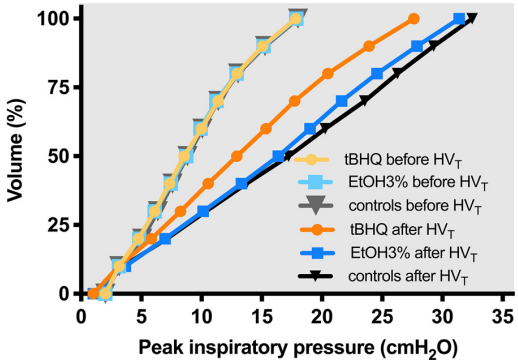

Figure 5. Pressure-volume curves before and after high tidal volume (HV_T) ventilation. Mice were pretreated with phosphate-buffered saline (controls, n = 9), 3% ethanol (EtOH3%, n = 13), or *tert*-butylhydroquinone (tBHQ, n = 9) and then subjected to HV_T ventilation. Pressure-volume curves were plotted as the inflation volume (expressed as a fraction of inspiratory capacity) as a function of airway pressure before and after HV_T ventilation. Deterioration of respiratory mechanics is indicated by a rightward shift of the pressure-volume curves compared with the curves at the beginning. Pressure-volume curves are displayed as the mean of all curves in each experimental group. For clarity, only the mean values are displayed. Missing samples: 1 control and 1 tBHQ due to technical failure to record pressure-volume curves.

tBHQ attenuates the development of lung edema and improves respiratory system compliance in mice subjected to HV_T ventilation.

The total protein concentration in the bronchoalveolar lavage fluid (BALF)—a surrogate for the degree of lung edema—was low in mice subjected to LV_T ventilation (Fig. 4*A*, 0.2 ± 0.02 μg/μL). HV_T ventilation among controls caused a 10-fold increase of protein concentration in BALF (Fig. 4*A*, 2 ± 0.7 μg/μL, $P < 0.001$ LV_T vs. HV_T controls). Pretreatment with tBHQ resulted in a total protein concentration in BALF that was 35% lower (Fig. 4*A*, 1.3 ± 0.6 μg/μL, P = 0.093 tBHQ vs. controls), but EtOH3% had no effect (Fig. 4*A*, 1.9 ± 0.6 μg/μL, $P > 0.999$ EtOH3% vs. controls). Analysis of potential correlations between the total protein concentration in BALF and the duration of HV_T ventilation in all groups revealed that a strong negative correlation exists in the tBHQ subgroups only (Fig. 4*B*, r = −0.8923, P = 0.001).

Dynamic and semistatic respiratory system compliances were comparable between all groups at baseline (Table 1). In controls, dynamic compliance decreased approximately by 35% and semistatic compliance by 50% after HV_T ventilation (Table 1). The reduction of respiratory system compliance was also illustrated by a rightward shift of the pressure-volume curve after HV_T ventilation (Fig. 5, *black line*).

Pretreatment with tBHQ reduced the HV_T ventilation-associated decrease of respiratory system compliance (Table 1) and attenuated the rightward shift of the pressure-volume curve after HV_T ventilation (Fig. 5, *orange*

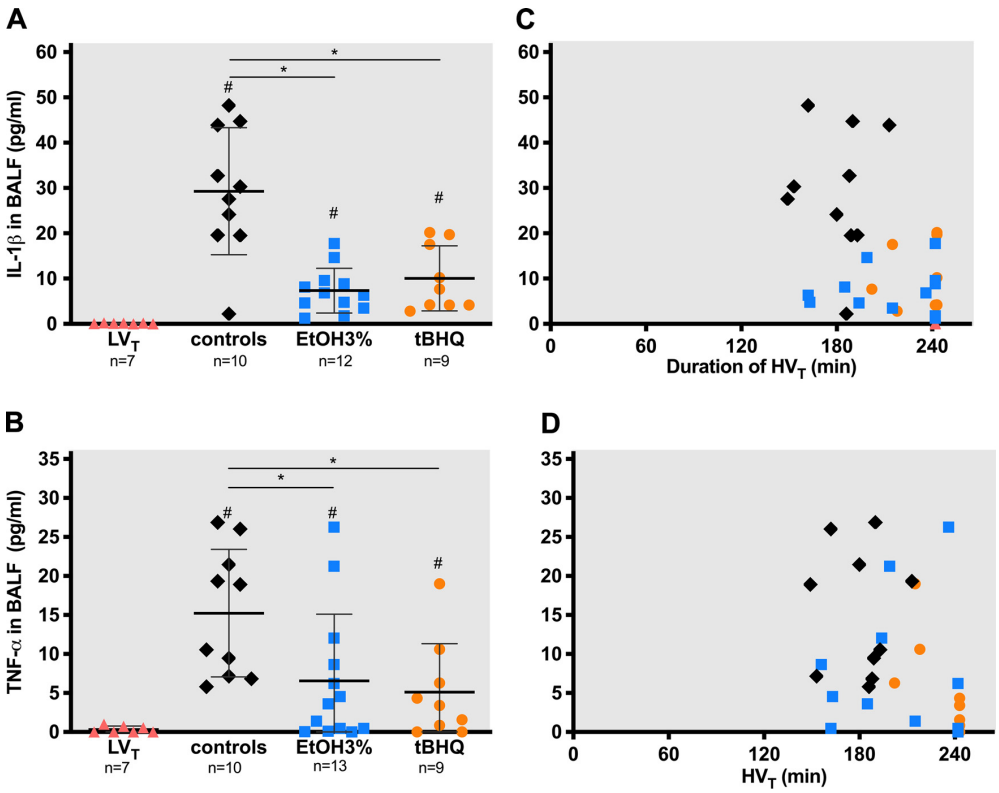

Figure 6. Concentrations of interleukin 1-β (IL-1β) (*A* and *C*) and tumor necrosis factor-α (TNF-α) (*B* and *D*) in the bronchoalveolar lavage fluid (BALF) after high tidal volume (HV_T) ventilation. Mice were pretreated with phosphate-buffered saline (controls, $n = 9$), 3% ethanol (EtOH3%, $n = 12$ in IL-1β and $n = 13$ in TNF-α), or *tert*-butylhydroquinone (tBHQ, $n = 10$) and then subjected to HV_T ventilation. A separate group of mice were pretreated with phosphate-buffered saline and subjected to low tidal volume ventilation for 5 h (LV_T, $n = 7$). BALF was collected at the end of the experiment. $*P < 0.05$ tBHQ/EtOH3% vs. controls (Kruskal–Wallis test). $\#P < 0.05$ LV_T vs. HV_T (unpaired Student's *t* test/Mann–Whitney *U* test). Missing samples: 1 tBHQ (failure to collect BALF) and 1 EtOH3% (not enough BALF). Two scatter plots were created to depict possible correlations between the concentrations of IL-1β (*C*) and TNF-α (*D*) in BALF and the duration of HV_T ventilation. No correlations were found for either IL-1β (*C*) or TNF-α (*D*) in any of the groups.

line). Pretreatment with EtOH3% had little effect on respiratory system compliance compared with controls (Table 1) and caused a rightward shift of the pressure-volume curve comparable with the controls (Fig. 5, *blue line*).

tBHQ and EtOH3% ameliorate the proinflammatory response in the lung subjected to HV_T ventilation.

In mice subjected to LV_T ventilation, levels of IL-1β (Fig. 6*A*, 0.1 ± 0.08 pg/mL) and TNF-α (Fig. 6*B*, 0.3 ± 0.43 pg/mL) remained at a very low level as compared with all other groups. After HV_T ventilation, 29 ± 14 pg/mL of IL-1β and 15 ± 8 pg/mL of TNF-α were detected in BALF (Fig. 6, controls). Pretreatment with tBHQ as well as EtOH3% resulted in an approximately threefold reduction of these proinflammatory cytokines IL-1β (Fig. 6*A*, $P = 0.030$ tBHQ vs. controls, $P = 0.002$ EtOH3% vs. controls) and TNF-α in BALF (Fig. 6*B*, $P = 0.025$ tBHQ vs. controls, $P = 0.031$ EtOH3% vs. controls). Concentrations of IL-1β and TNF-α showed no correlation with the duration of HV_T ventilation (Fig. 6, *C* and *D*).

HV_T ventilation was associated with activation of the inflammatory canonical pathway of nuclear factor kappa B (NF-κB), reflected by the phosphorylated p65/total p65 ratios in the lung tissue. Pretreatment with tBHQ and EtOH3% decreased the phosphorylated p65/total p65 ratios in the lung tissue after HV_T ventilation (Fig. 7, $P = 0.0235$ tBHQ vs. controls, $P = 0.007$ EtOH3% vs. controls) to a level comparable with LV_T ventilation.

tBHQ boosts pulmonary antioxidant Nrf2-dependent gene expression at baseline and augments total concentration of glutathione and NRF2 protein in lung tissue after HV_T ventilation.

To assess the antioxidant capacity of the lungs before HV_T ventilation (at baseline), we measured the Nrf2-dependent gene expression, NRF2 protein concentration as well as total concentration of glutathione, and the reduced/oxidized glutathione ratio (GSH/GSSG) in the lungs of mice euthanized 1 h after pretreatment and not subjected to HV_T ventilation.

Pretreatment with tBHQ yielded a 1.7-fold increase of the relative expression of pulmonary messenger RNA (mRNA) of *Nrf2* (Fig. 8*A*, $P < 0.001$) and a 1.5-fold increase of *Sod1* (Fig. 8*B*, $P = 0.012$) compared with controls at baseline (no HV_T). EtOH3% had no effect on the relative expression of

Nrf2 (Fig. 8*A*) and Sod1 (Fig. 8*B*) at baseline. Neither tBHQ nor EtOH3% increased the relative expression of *Gclc* (Fig. 8*C*) or *Gclm* (Fig. 8*D*) compared with controls at baseline. Similarly, tBHQ and EtOH3% had no effect on the total concentration of NRF2 protein (Fig. 9*A*) and glutathione (Fig. 9*B*) as well as GSH/GSSG ratio (Fig. 10) at baseline.

Among controls, HV_T ventilation per se induced a 3–4-fold increase of relative pulmonary expression of *Nrf2* (Fig. 8*A*) and *Sod1* (Fig. 8*B*), as well as a 10-fold increase of *Gclc* (Fig. 8*C*) and a 5-fold increase of *Gclm* (Fig. 8*D*). HV_T ventilation also increased the concentration of NRF2 protein (Fig. 9*A*), total concentration of glutathione (Fig. 9*B*), and GSH/GSSH ratio (Fig. 10) in the lung tissue.

Mice pretreated with tBHQ or EtOH3% that were subjected to HV_T ventilation yielded a similar increase of Nrf2-dependent gene expression of *Nrf2* (Fig. 8*A*), *Sod1* (Fig. 8*B*), and *Gclc* (Fig. 8*C*) compared with controls (HV_T). However, compared with controls, tBHQ boosted the pulmonary gene expression of *Gclm* in mice subjected to HV_T ventilation (Fig. 8*D*, P = 0.014). Likewise, pretreatment with tBHQ augmented the NRF2 protein concentration in lung tissue after HV_T ventilation as compared with controls (Fig. 9*A*) and provided the highest total pulmonary glutathione concentrations (Fig. 9*B*, 48 ± 8 μmol/g tissue) as compared with controls (36 ± 4 μmol/g tissue, P = 0.004) and EtOH3% (Fig. 9*B*, 41 ± 6 μmol/g tissue, P = 0.040). The GSH/GSSG ratio was similar among controls and mice pretreated with tBHQ or EtOH3% (Fig. 10, HV_T).

Hemodynamic and ventilatory parameters during HV_T ventilation.

No differences in mean arterial blood pressure, pulse rate, peak inspiratory pressure, or tidal volume were observed between groups at the beginning of HV_T ventilation (Figs. 11 and 12). During HV_T ventilation, a gradual decrease of blood pressure (Fig. 11, *A* and *C*) and tidal volume (Fig. 12, *B* and *D*) occurred, as well as a gradual increase in peak inspiratory pressure (Fig. 12, *A* and *C*). Pulse rate remained relatively constant throughout the experiment (Fig. 11, *B* and *D*). Animals not alive at 240 min of HV_T ventilation had a higher peak inspiratory pressure at the end of HV_T ventilation compared with those who completed 240 min alive (Fig. 13, 39 ± 0.7 cmH_2O vs. 33 ± 2.4 cmH_2O, $P < 0.001$).

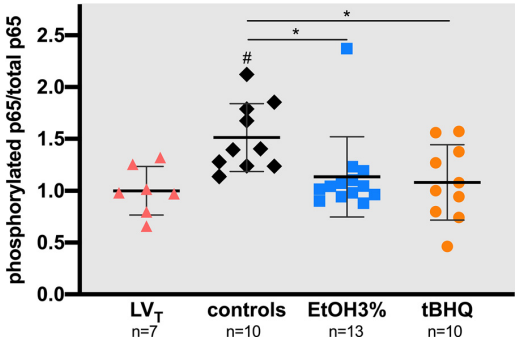

Figure 7. Phosphorylated p65 and total p65 ratio in lung tissue after high tidal volume (HV_T) ventilation. Mice were pretreated with phosphate-buffered saline (controls, n = 10), 3% ethanol (EtOH3%, n = 13), or *tert*-butylhydroquinone (tBHQ, n = 10) and subjected to HV_T ventilation. A separate group of mice were pretreated with phosphate-buffered saline and subjected to low tidal volume ventilation for 5 h (LV_T, n = 7). P65 was quantified with an ELISA in lung tissue collected at the end of the experiment. *$P < 0.05$ tBHQ/EtOH3% vs. controls (Kruskal–Wallis test). #$P < 0.05$ LV_T vs. HV_T (unpaired Student's t test/Mann–Whitney U test).

DISCUSSION

In this study, we tested whether the pharmacological activation of the Nrf2-ARE pathway by tBHQ protects against the development of VILI in a mouse model of HV_T ventilation. We found that pretreatment with tBHQ improved survival during HV_T ventilation and reduced the deterioration of respiratory system compliance as well as arterial blood oxygenation by attenuating the development of lung edema. This protective effect was associated with increased antioxidant capacity as well as reduced inflammation in the lung.

The protective effect of tBHQ against VILI was associated with increased antioxidant capacity of the lungs—tBHQ increased antioxidant gene expression in the lungs before mechanical ventilation and provided the highest levels of total glutathione after HV_T ventilation. In line with studies that have shown the role of oxidative stress in the development of pulmonary endothelial hyperpermeability, our data suggest that protection against oxidative stress by increasing the antioxidant capacity could play an important role in preserving the integrity of the epithelial-endothelial barrier (3, 22).

More precisely, our data show that tBHQ increases the Nrf2-dependent gene expression of *Nrf2* and *Sod1* but not the pulmonary NRF2 protein concentration at baseline, i.e., 1 h after the injection of tBHQ and before the initiation of mechanical ventilation. However, tBHQ pretreatment resulted in higher NRF2 protein concentrations in the lung after HV_T ventilation. A putative mismatch between the increased *nrf2* gene expression and the NRF2 protein concentrations induced by tBHQ at 1 h after the intraperitoneal injection (baseline) may reflect the differential time intervals required for gene expression versus protein synthesis.

This is an important finding, suggesting that tBHQ preconditioned the lung for imminent oxidative stress by increasing antioxidant gene expression and thereby increasing the antioxidant capacity of the pulmonary cells before mechanical ventilation. Increased Nrf2-dependent gene expression has been associated with the protective effect of tBHQ against tissue damage in the kidney and liver of rats exposed to ischemia-reperfusion injury (17, 35). Moreover, other authors have shown that Nrf2-deficient mice developed more severe VILI and that supplementation with antioxidant *N*-acetyl-L-cysteine (NAC) attenuated the disadvantages of Nrf2-deficiency, concluding that the physiological functionality of the Nrf2-ARE pathway is crucial for the severity of VILI (31). Applicable to the physiological protective role of the Nrf2-ARE pathway in the resilience against VILI, our study adds evidence that pharmacological activation of the Nrf2-ARE pathway by tBHQ could be exploited as a therapy to increase protection against VILI.

Glutathione is a major antioxidant in mammalian cells and its biosynthesis is dependent on glutamate-cysteine ligase (GLC) that is linked to the activation of the Nrf2-ARE

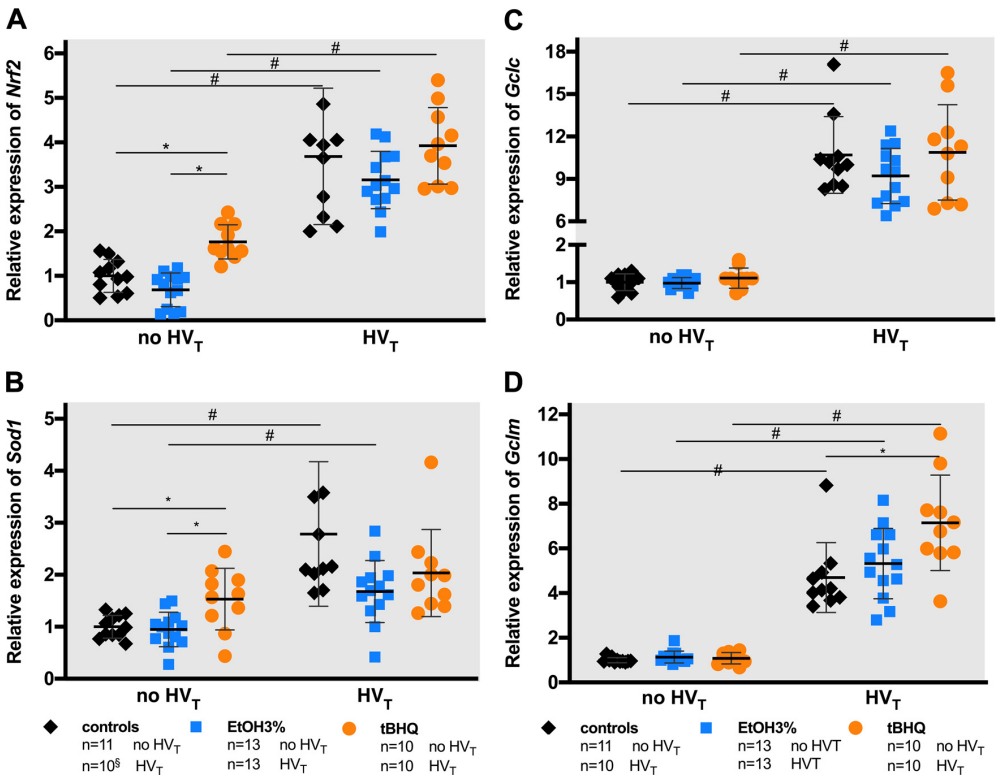

Figure 8. Pulmonary messenger RNA (mRNA) concentrations of *Nrf2* (*A*), *Sod1* (*B*), *Gclc* (*C*), and *Gclm* (*D*). Mice were pretreated with phosphate-buffered saline (controls), 3% ethanol (EtOH3%), or *tert*-butylhydroquinone (tBHQ) and either euthanized 1 h later (no HV_T) or subjected to high tidal volume ventilation (HV_T). mRNA concentrations are expressed as fold change relative to the average expression values in no HV_T controls. $^*P < 0.05$ tBHQ vs. controls/EtOH3% among no HV_T (one-way ANOVA), $\#P < 0.05$ no HV_T vs. HV_T (unpaired Student's *t* test/Mann–Whitney *U* test). §1 outlier for *Nrf2* and 2 outliers for *Sod1* were included in the statistical analysis but are not depicted on this graph to increase visual comprehension.

pathway (12). Functionally, GLC is a holoenzyme consisting of a catalytic (GCLC) and a modifier (GCLM) subunit, encoded by two separate genes (*Gclc* and *Gclm*). GCLM is enzymatically inactive, but GLC is much more efficient and less prone to inhibition by GSH in the presence of GCLM (25). We observed that the relative expression of *Gclc* and *Gclm* was comparable in all groups before mechanical ventilation and that HV_T ventilation per se induced the expression of both genes. Interestingly, pretreatment with tBHQ boosted the expression of *Gclm* but not *Gclc* in mice subjected to HV_T ventilation. This is in line with other studies showing that tBHQ more strongly increases the expression of *Gclm* (33).

Several authors have shown that glutathione is associated with protection against VILI (8, 13, 31). Our data show that pretreatment with tBHQ resulted in the highest concentration of total glutathione in the lung tissue after HV_T ventilation as compared with controls and EtOH% and provided a clinically significant protection against VILI. Considering that cyclic stretching of lung cells typically induces an initial reduction of glutathione (consumption) followed by a reactive increase (synthesis) (19), we believe that tBHQ pretreatment augmented Nrf2-dependent gene expression of *Gclm* early enough (preconditioning) to result in more efficient glutathione production during subsequent HV_T ventilation.

Considering the induction of Nrf2-dependent gene expression, it is important to note that the mechanical stimulus of HV_T ventilation had a greater impact on the antioxidative response than the pharmacological stimulation with tBHQ at baseline. The increase of Nrf2-dependent gene expression in the lung was three- to fourfold in all groups after HV_T ventilation. Presumably, this reflects an innate response to mechanical stretch-induced oxidative stress, activating the Nrf2-ARE pathway and promoting a positive feedback mechanism between mechanical stretch, oxidative stress, and antioxidant gene expression (6, 7, 31). Tidal volumes of ~40 mL/kg that were applied in the present study exceeded the total lung capacity and caused highly injurious and ultimately lethal dynamic stretch and strain (32, 38). Sun et al. (34) suggested that a proportional relationship between the size of the tidal volume, the magnitude of oxidative stress, and the level of antioxidative responses exists: they observed that rats ventilated with higher tidal volumes also had higher concentrations of malondialdehyde and expression of *Nrf2* in the lung tissue. Such a relationship supports our finding that tidal volumes of 40mL/kg were associated with pronounced activation of antioxidative responses in all groups receiving HV_T ventilation. The level of "mechanically induced" Nrf2-ARE pathway activation and antioxidative response brought about by HV_T ventilation easily exceeded

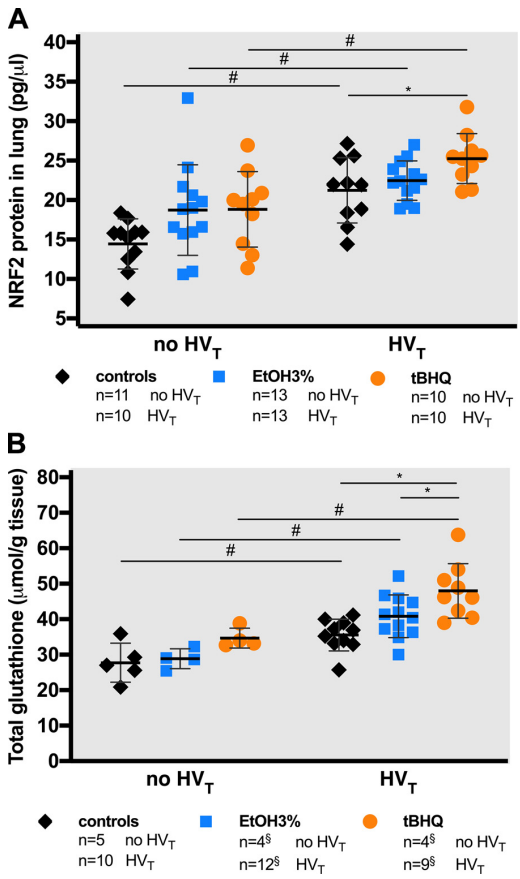

Figure 9. NRF2 protein (*A*) and total glutathione (*B*) concentration in lung tissue. Mice were pretreated with phosphate-buffered saline (controls), 3% ethanol (EtOH3%), or *tert*-butylhydroquinone (tBHQ) and either euthanized 1 h later (no HV_T) or subjected to high tidal volume (HV_T) ventilation. *$P < 0.05$ tBHQ vs. controls/EtOH3% among HV_T (one-way ANOVA). #$P < 0.05$ no HV_T vs. HV_T (unpaired Student's *t* test/Mann–Whitney *U* test). §1 extreme outlier was excluded from analysis.

the level of pharmacological activation induced by tBHQ at baseline.

In the context of biotrauma, it is important to note that the protective effects of tBHQ against VILI were also associated with a decreased proinflammatory response to HV_T ventilation in the lungs. We found an approximately threefold reduction of the concentrations of proinflammatory cytokines IL-1β and TNF-α in BALF of mice pretreated with tBHQ. Interestingly, a similar reduction of the proinflammatory response to HV_T ventilation was found in mice pretreated with EtOH3%, the solvent for poorly water-soluble tBHQ. However, although EtOH3% reduced the concentrations of proinflammatory cytokines after HV_T ventilation to a similar extent, the therapeutic effects of EtOH3% were inferior to tBHQ. Only tBHQ improved arterial blood oxygenation and attenuated the deterioration of respiratory system compliance associated with lung edema after HV_T ventilation. Development of lung edema due to increased permeability of the pulmonary epithelial-endothelial barrier is a characteristic of VILI that indicates structural and functional damage of the lung parenchyma. Pretreatment with tBHQ protected more comprehensively against biotrauma from HV_T ventilation by increasing the antioxidant capacity of the lungs.

Finally, pretreatment with EtOH3% also improved survival after HV_T ventilation but not to the extent of tBHQ. These findings underscore the importance of vehicle control groups, to separate the effects of the target compound (e.g., tBHQ) and its specific solvent (e.g., EtOH3%).

A hallmark of VILI, pulmonary inflammation can contribute to mortality by promoting systemic inflammation and thereby triggering vasoplegic shock (24, 40). Human data also point to the relevance of systemic inflammation in acute lung injury. In patients with ARDS, systemic inflammation contributes to the risk of multiple-organ dysfunction and is associated with poor outcome (26). In addition, the ARDS-Network study that compared lower tidal volumes with traditional volumes showed that the reduced mortality of patients ventilated with the protective strategy was associated with lower concentrations of proinflammatory cytokines in the plasma (5).

Notably, our data show that pretreatment with both tBHQ and EtOH3% was associated with the activation of the canonical pathway of nuclear factor kappa b (NF-κB) in mice after HV_T ventilation. The canonical pathway of NF-κB is commonly associated with inflammatory responses (2). We believe that in our study, tBHQ and EtOH3% might have synergistically affected the NF-κB pathway. First, pretreatment with tBHQ has been shown to reduce the activation of NF-κB in mouse models of traumatic brain and spinal cord injury (20, 21). Second, other authors have found similar NF-κB activation in human lung epithelial cells exposed to 1% ethanol (28).

Reflecting the detrimental impact of systemic inflammation and vasoplegic shock on the outcome of lung injury, mean arterial pressure below 40 mmHg predicted imminent circulatory arrest (termination trigger) (29) in our model and was used as an end point to define mortality. We observed a

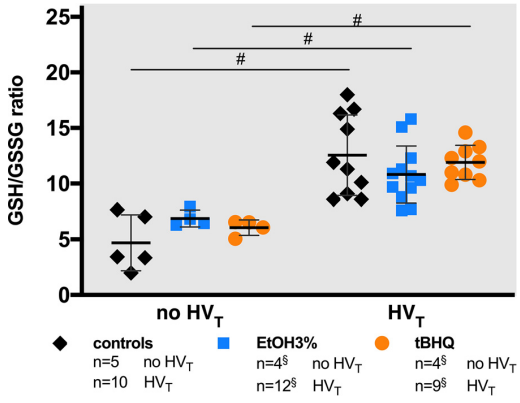

Figure 10. Reduced glutathione (GSH) and oxidized glutathione (GSSG) ratio in lung tissue. Mice were pretreated with phosphate-buffered saline (controls), 3% ethanol (EtOH3%), or *tert*-butylhydroquinone (tBHQ) and either euthanized 1 h later (no HV_T) or subjected to high tidal volume (HV_T) ventilation. #$P < 0.05$ no HV_T vs. HV_T (unpaired Student's *t* test/Mann–Whitney *U* test). §1 extreme outlier was excluded from analysis.

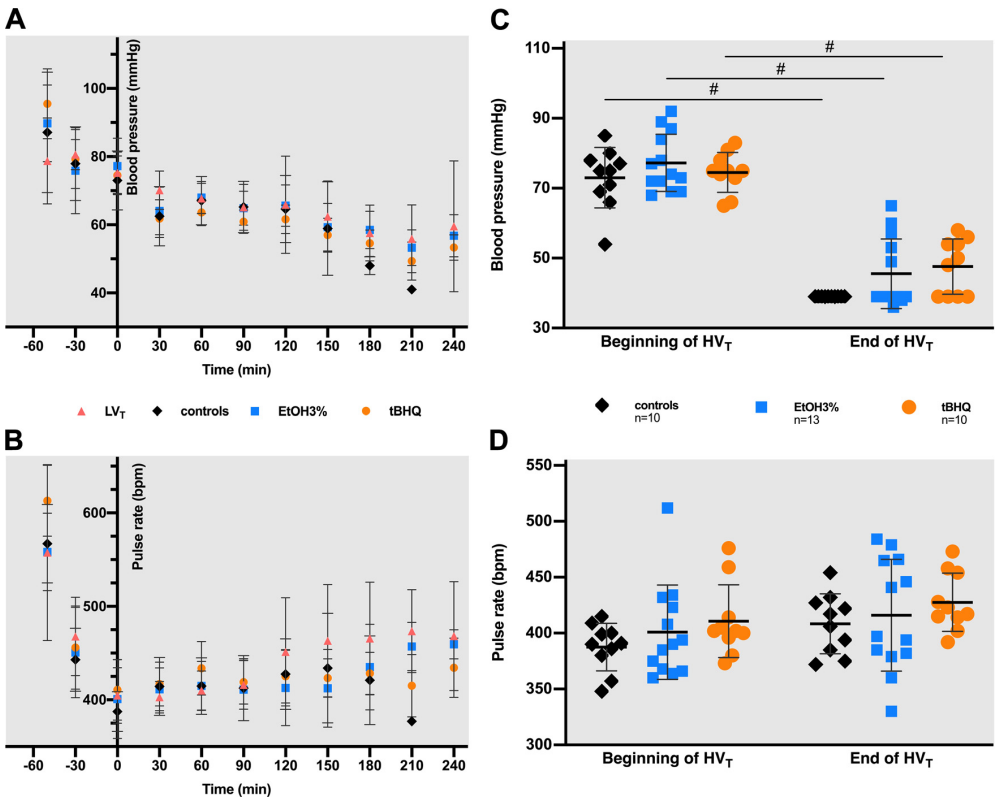

Figure 11. Hemodynamic parameters. Mice were pretreated with phosphate-buffered saline (controls, $n = 10$), 3% ethanol (EtOH3%, $n = 13$), or *tert*-butylhydroquinone (tBHQ, $n = 10$) and subjected to HV_T ventilation. A separate group of mice were pretreated with phosphate-buffered saline and subjected to low tidal volume ventilation for 5 h (LV_T, $n = 7$). *Graphs A* and *B* depict the mean arterial blood pressure (mmHg) and pulse rate (bpm) measured at 30 min intervals during baseline ventilation (V_T 10 mL/kg) before T_0 and during subsequent low (10 mL/kg) or high tidal volume (40 mL/kg) ventilation after T_0 for up to 240 min. During HV_T ventilation, mice gradually dropped out from the analysis as they died. *Graphs C* and *D* depict the mean arterial blood pressure (mmHg) and pulse rate (bpm) measured at the beginning and at the end of high tidal volume ventilation. #$P < 0.05$ before HV_T vs. after HV_T (two-way repeated-measures ANOVA).

gradual drop in blood pressure during the course of HV_T ventilation with a relatively stable pulse rate (Fig. 11), up until the termination trigger was reached. We believe that mortality was due to cardiopulmonary deterioration, including an interdependence of hypoxemia, hypotension, and cardiac failure. Of note, mice that did not survive 240 min of HV_T ventilation had developed extremely high peak inspiratory pressures (Fig. 13). High peak inspiratory pressure and hypoxic pulmonary vasoconstriction can increase pulmonary vascular resistance significantly, resulting in right heart dysfunction (36). Major respiratory swings from high peak pressure and low PEEP have also been shown to cause right heart failure likely due to pulmonary microvascular injury (23). Right heart dysfunction is also considered one of the major contributors for mortality in patients with ARDS (4). We therefore believe that the progression of alveolar edema that deteriorates pulmonary compliance, resulting in high peak airway and intrathoracic pressures and causing hypoxemia, was a major component of a vicious cycle contributing to hypoxic pulmonary vasoconstriction, increased right ventricular afterload, and right heart failure. Under these circumstances, systemic inflammation and vasoplegia—even at a low level—may significantly contribute to the rapidity of these events. Based on these considerations, we believe that the survival benefit produced by tBHQ and EtOH3% in the present study is attributable to reduced pulmonary and likely systemic inflammation. Reduction of pulmonary inflammation and oxidative stress decelerates formation of lung edema, delaying the series of events causing cardiocirculatory depression from hypoxemia.

This study has several limitations. First, we used higher tidal volumes than those commonly used in clinical practice. However, in patients with ARDS, who have relevant lung heterogeneity as well as a small functional lung volume (the "baby lung" concept), relatively small tidal volumes can cause extreme regional overdistention (9, 15). In addition, tidal volumes of ~40mL/kg on a healthy mouse resulted in a PIP of 30 cmH_2O, a level that, in the clinical setting, is considered a threshold to initiate rescue therapies such as extracorporeal membrane oxygenation (ECMO). Second, although the etiology of acute lung injury in the clinical setting is usually multifactorial, the model we used in this study primarily reflects a single injurious hit emanating from ventilation-induced alveolar overdistention. Third, this study did not include the measurement of systemic inflammatory markers, pulmonary

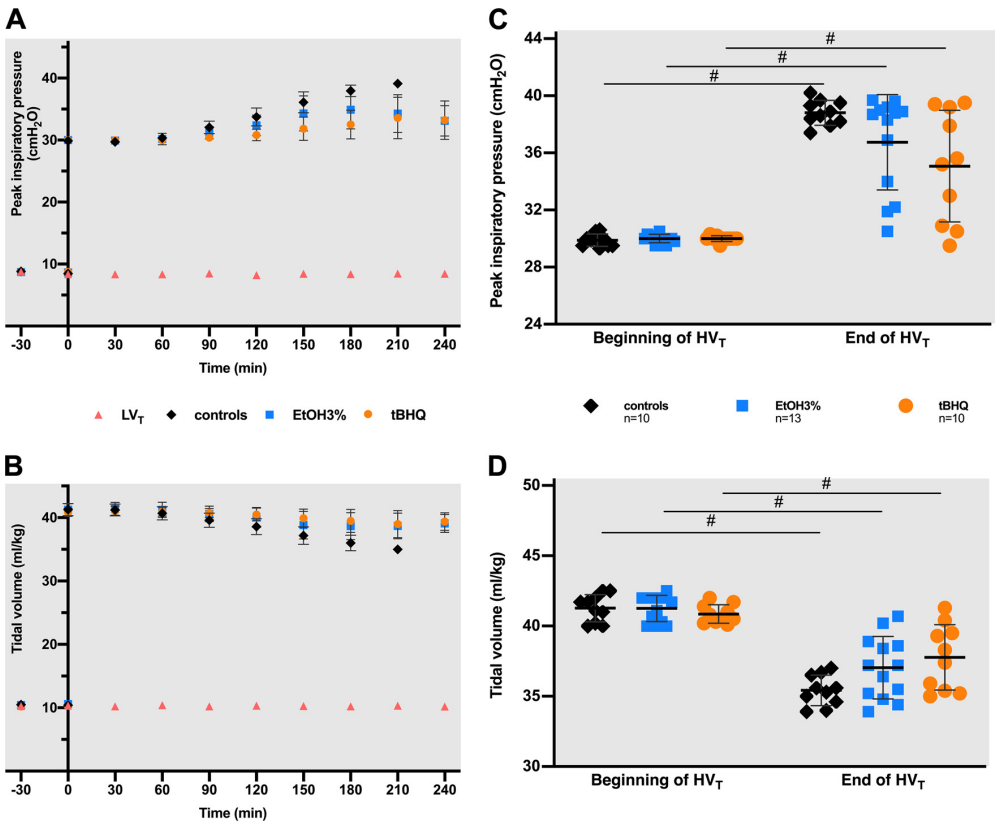

Figure 12. Ventilatory parameters. Mice were pretreated with phosphate-buffered saline (controls, *n* = 10), 3% ethanol (EtOH3%, *n* = 13), or *tert*-butylhydroquinone (tBHQ, *n* = 10) and subjected to HV_T ventilation. A separate group of mice were pretreated with phosphate-buffered saline and subjected to low tidal volume ventilation for 5 h (LV_T, *n* = 7). *Graphs A* and *B* depict the peak inspiratory pressure (cmH_2O) and tidal volume (mL/kg) measured at 30 min intervals during baseline ventilation (V_T 10 mL/kg) before T_0 and during subsequent low (10 mL/kg) or high tidal volume (40 mL/kg) ventilation after T_0 for up to 240 min. During HV_T ventilation, mice gradually dropped out from the analysis as they died. *Graphs C* and *D* depict the peak inspiratory pressure (cmH_2O) and tidal volume (mL/kg) measured at the beginning and at the end of high tidal volume. #$P < 0.05$ before HV_T vs. after HV_T (two-way repeated-measures ANOVA).

vascular resistance, or cardiac output, to elucidate more details of the causal and temporal contributions of these parameters to the lethality of this model.

SUMMARY

This study demonstrates that the Nrf2-ARE pathway activator tBHQ improves survival and attenuates the development of pulmonary inflammation, lung edema, and respiratory system dysfunction of mice in a lethal one-hit model of VILI induced by mechanical ventilation with high tidal volumes. The protective effect of tBHQ is associated with increased pulmonary Nrf2-dependent antioxidant gene expression and can be considered an antioxidant preconditioning of the lung parenchyma. These findings support the efficacy of pharmacological activation of the Nrf2-ARE pathway and of potential novel drug targets to increase the physiological resilience against oxidative stress responses during mechanical ventilation with risk of VILI.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

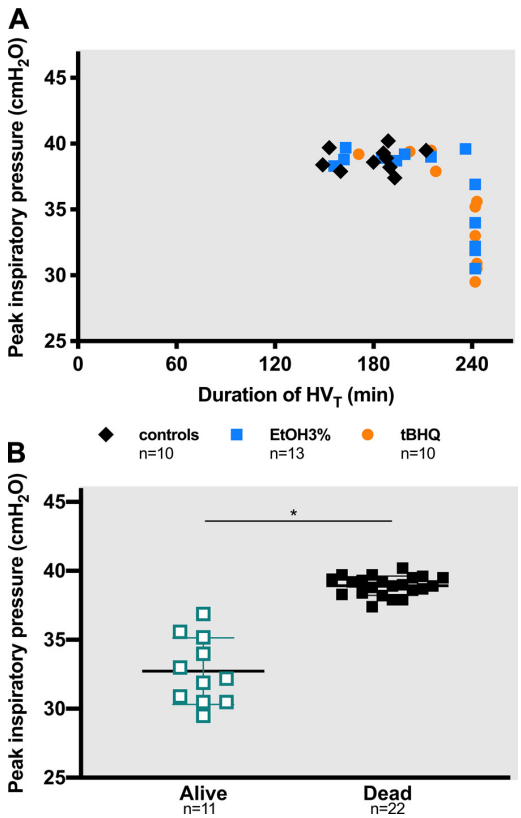

Figure 13. Peak inspiratory pressure at the end of the experiment. Mice were pretreated with phosphate-buffered saline (controls, n = 10), 3% ethanol (EtOH3%, n = 13), or *tert*-butylhydroquinone (tBHQ, n = 10) and subjected to HV_T ventilation. Graph *A* is a scatter plot depicting peak inspiratory pressure (PIP, cmH_2O) at the end of the experiment. On graph *B*, the mice are regrouped. Animals that survived 240 min of HV_T ventilation were included in group *Alive* and animals that died before 240 min of HV_T ventilation were included in group *Dead*. *$P < 0.0001$ *Alive* vs. *Dead* (unpaired Student's *t* test).

AUTHOR CONTRIBUTIONS

J.A.G. and R.C.E.F. conceived and designed research; L.V. and A.G.-L., performed experiments; L.V., A.G.-L., and R.C.E.F. analyzed data; L.V., J.A.G., P.A.P., M.T., W.B., A.G.-L., and R.C.E.F. interpreted results of experiments; L.V. prepared figures; L.V. drafted manuscript; L.V., J.A.G., P.A.P., M.T., W.B., A.G.-L., and R.C.E.F. edited and revised manuscript; L.V., J.A.G., P.A.P., M.T., W.B., A.G.-L., and R.C.E.F. approved final version of manuscript.

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12. Curriculum Vitae

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht

13. List of Publications

Veskemaa L, Graw JA, Pickerodt PA, Taher M, Boemke W, Gonzalez-Lopez A, and Francis RCE. Tert-butylhydroquinone augments Nrf2-dependent resilience against oxidative stress and improves survival of ventilator-induced lung injury in mice. *Am J Physiol Lung Cell Mol Physiol* 2020.
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13.1. Poster presentations at scientific conferences

Lilly Veskemaa, Adrian Gonzalez-Lopez, Philipp Pickerodt, Birgit Brandt, Jan Adriaan Graw, Willehad Boemke, Roland C.E. Francis. Nrf2-antioxidant signalling pathway activator tBHQ protects mice against ventilator-induced lung injury. Poster presentation at the Hauptstadtkongress der DGAI für Anästhesiologie und Intensivmedizin, September 20-22, 2018 in Berlin.

Lilly Veskemaa, Adrian Gonzalez-Lopez, Philipp Pickerodt, Birgit Brandt, Jan Adriaan Graw, Willehad Boemke, Roland C.E. Francis. Nrf2-antioxidant signalling pathway activator tBHQ protects mice against ventilator-induced lung injury. Poster presentation at the 5th International ARDS Conference, June 25-27, 2019 in Berlin.

Lilly Veskemaa. Supervisor: Jaanus Kahu MD/PhD. Perioperative antibiotic prophylactic in kidney transplantation. Poster presentation at a student conference in University of Tartu, 2011 in Tartu.

Lilly Veskemaa et.al. Supervisor: Katrin Sonn MD. 5xFAD mice as a animal model for Alzheimer's disease. Poster presentation at a student conference in University of Tartu, 2010 in Tartu.

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