# The fall in exhaled nitric oxide with ventilation at low lung volumes in rabbits: an index of small airway injury

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**Abstract**

The mechanisms involved in the fall of exhaled nitric oxide (NOe) concentration occurring in normal, anesthetized open chest rabbits with prolonged mechanical ventilation (MV) at low lung volume have been investigated. NOe, pH of exhaled vapor condensate, serum prostaglandin $E_2$, and $F_{2\alpha}$, tumor necrosis factor (TNF-α), $PaO_2$, $PaCO_2$, pHa, and lung mechanics were assessed before, during, and after 3-4 h of MV at zero end-expiratory pressure (ZEEP), with fixed tidal volume (9 $ml \cdot kg^{-1}$) and frequency, as well as before and after 3-4 h of MV on PEEP only. Lung histology and wet-to-dry ratio (W/D), and prostaglandin and TNF-α in bronchoalveolar lavage fluid (BALF) were also assessed. While MV on PEEP had no effect on the parameters above, MV on ZEEP caused a marked fall (45%) of NOe, with a persistent increase of airway resistance (45%) and lung elastance (12%). Changes in NOe were independent of prostaglandin and TNF-α levels, systemic hypoxia, hypercapnia and acidosis, bronchiolar and alveolar interstitial edema, and pH of exhaled vapor condensate. In contrast, there was a significant relationship between the decrease in NOe and bronchiolar epithelial injury score. This indicates that the fall in NOe, which occurs in the absence of an inflammatory response, is due to the epithelial damage caused by the abnormal stresses related to cyclic opening and closing of small airways with MV on ZEEP, and suggests its use as a sign of peripheral airway injury.

Keywords: low volume ventilation; lung mechanics; nitric oxide; prostaglandins; small airway injury

## 1. Introduction

In normal, anesthetized, paralyzed, open-chest rabbits, prolonged mechanical ventilation at low lung volumes with physiological tidal volumes causes bronchiolar epithelial damage and rupture of alveolar septa connected to peribronchiolar membrane, with a concomitant increase in airway resistance which persists after restoration of physiological end-expiratory lung volume (D'Angelo et al., 2002; 2004). Furthermore, an increased number of granulocytes was observed in the alveolar septa (D'Angelo et al., 2004), suggesting the presence of parenchymal inflammation. Both types of injury can be responsible for the persistent increase of pulmonary resistance, altering the bronchiolar lumen and the bronchiolar-alveolar coupling, and causing the release of inflammatory mediators with bronchoconstrictor effects. It has been recently shown that these histologic and functional alterations are the consequence of abnormal stresses that develop with cyclic opening and closing of peripheral airways during tidal ventilation at low volumes, because they are prevented by the administration of exogenous surfactant and worsened by artificially induced dysfunction of the natural surfactant (D'Angelo et al., 2007).

Prolonged mechanical ventilation at low lung volume in normal, anaesthetized, paralyzed rabbits, both closed and open chest, has been also shown to cause a reduction of nitric oxide concentration in the exhaled air (NOe) that persists after restoration of physiological end-expiratory volumes (D'Angelo et al., 2005). It was suggested that the fall in NOe could be due to epithelial damage, but no direct observations were made which could support the presence of a relation between levels of NOe and extent of small airway injury. On the other hand, other events could be also responsible for the fall in NOe, such as release of prostaglandin $E_2$ and $F_{2\alpha}$ from the alveolar epithelium, macrophages, and granulocytes activated by mechanical insults (Delgado et al., 1999; Kharitonov et al., 1999), development of bronchiolar edema (Cremona et al., 1995), and changes in pH of the fluid lining the airway epithelium (Hunt et al., 2000). The aim of the present research is therefore that of assessing the mechanisms involved in the reduction of NOe with mechanical ventilation at low lung volume. To this end, we have studied in open chest rabbits the dependence of NOe on the levels of $PGE_2$ and $PGF_{2\alpha}$ in serum and bronchoalveolar lavage fluid, extent of interstitial edema, pH of the condensate, as well as the relation between the fall of NOe and histologic indices of lung damage. The results have indicated that damage of small airway epithelium is the most likely cause of the reduction of NOe levels, which could be, therefore considered as a sign of peripheral airway injury

## 2. Methods

Twenty-four New Zealand white rabbits (weight range: 2-3.3 kg) were anesthetized with an intravenous injection of a mixture of pentobarbital sodium (20 $mg \cdot kg^{-1}$) and urethane (0.5 $mg \cdot kg^{-1}$). A brass cannula and polyethylene catheters were inserted into the trachea, the carotid and femoral artery, and the external jugular vein, respectively. The animals were paralyzed with pancuronium bromide (0.1 $mg \cdot kg^{-1}$) and mechanically ventilated (respirator 660; Harvard Apparatus, Holliston, MA) with a pattern similar to that during spontaneous breathing. Anesthesia and complete muscle relaxation were maintained with additional doses of the anesthetic mixture and pancuronium bromide. Adequacy of anesthesia was judged from the absence of mydriasis, sudden increase of systemic blood pressure and/or heart rate. Throughout the experiment, Ringer-bicarbonate solution was infused via the jugular vein at a rate of 4 $ml \cdot kg^{-1} \cdot h^{-1}$. The chest was opened via a median sternotomy, a coronal cut was made just above the costal arch, and a positive end-expiratory pressure of 2-2.5 $cmH_2O$ was applied. During the mechanics measurements, the ribs on the two sides and the diaphragm were pulled widely apart, in order to prevent contact between lung and chest wall, except in their dependent parts. The animals rested supine on a heating pad; rectal temperature was kept under all conditions at 37±0.1 °C. The investigation was authorized by the Italian Ministry of Health.

Airflow ($\dot{V}$) was measured with a heated Fleisch pneumotachograph no.00 (HS Electronics, March-Hugstetten, Germany) connected to the tracheal cannula and a differential pressure transducer (Validyne MP45, ±2 $cmH_2O$; Northridge, CA). The response of the pneumotachograph was linear over the experimental range of $\dot{V}$. Tracheal (Ptr) and systemic blood pressure (Pa) were measured with pressure transducers (model 1290A; Hewlett-Packard, Palo Alto, CA) connected to the side arm of the tracheal cannula and the femoral artery, respectively. There was no appreciable shift in the signals or alteration in amplitude up to 20 Hz. Nitric oxide (NO) was measured using a chemiluminescence analyzer (NOA 280i, Sievers, Boulder, CO), attached to a side port of the tracheal cannula through teflon tubing and set to draw air at a rate of 200 $ml \cdot min^{-1}$. Zeroing and calibration of the NO analyzer was verified repeatedly during the experiment using the Zero Air Filter® and certified gas mixture provided by the manufacturer. The signals from the transducers were amplified (model RS3800; Gould Electronics, Valley View, OH), sampled at 200 Hz by a 12-bit A/D converter (AT MIO16E-10; National Instruments, Austin, TX), and stored on a desk computer, together with the signal from the NO analyzer. Volume changes ($\Delta V$) were obtained by numerical integration of the digitized airflow signal. $Pa_{O_2}$, $Pa_{CO_2}$ and pHa were measured by means of a

blood gas analyzer (IL 1620; Instrumentation Laboratory, Milan, Italy), while the pH of the deaerated, exhaled airway vapor condensate was measured using an Amersham Pharmacia C900 pH meter (Uppsala, Sweden).

Analysis of $PGE_2$, $PGF_{2a}$, and tumor necrosis factor (TNF-α) was carried out in a blinded fashion on bronchoalveolar lavage fluid (BALF) and serum, using commercially available ELISA kits specific for rabbit ($PGF_{2\alpha}$ and $PGE_2$ Assay, R&D Systems Inc., Minneapolis, MN; BD Bioscience, Franklin Lakes, NJ for TNF-α). $PGE_2$, $PGF_{2a}$, and TNF-α color development was measured at 450 nm, 405 nm, and 405 nm, respectively (Titertek Multiskan MCC, Flow Laboratories, Milan, Italy), background absorbancy of blank wells being subtracted from the standards and samples prior to determination of the concentration. The lower limit of detection was 19.6 $pg \cdot ml^{-1}$, 3.05 $pg \cdot ml^{-1}$ and 10 $pg \cdot ml^{-1}$ for $PGE_2$, $PGF_{2\alpha}$, and TNF-α, respectively, in which case concentration was assumed to be nil. The albumin concentration of the BALF supernatant and serum obtained shortly before lung lavage was determined with a clinical chemistry analyzer (Bayer ADVIA 2004, Jeol, Japan for Bayer Diagnostics Europe, Dublin, Ireland ) at 596 nm using the bromocresol green method (Albumin reagent, Bayer, Tarrytown, UK) with bovine albumin as standard.

After completion of the surgical procedure and instrumentation, the rabbits were ventilated with a specially designed, computer-controlled ventilator (D'Angelo et al., 2002), delivering a NO free (NO concentration <0.5 ppb), water-saturated gas mixture from a high pressure source (4 atm) at constant flow of different selected magnitudes and duration. A three way stopcock allowed the connection of the expiratory valve of the ventilator either to the ambient (zero end-expiratory pressure; ZEEP) or to a drum in which the pressure could be made positive (positive end-expiratory pressure; PEEP) by means of a flow-through system. The baseline ventilator settings consisted of fixed tidal volume ($V_T$; 9 $ml \cdot kg^{-1}$), inspiratory duration ($T_I$; 0.25 s), and cycle duration (1.5 s). An end-inspiratory pause of 0.4 s was applied in order to ensure a normal mean lung volume during the respiratory cycle. During NO measurements both the inspiratory and expiratory duration were set at 1 s, the end-inspiratory pause was removed, and the $V_T$ was increased to compensate for the air drawn by the NO analyzer. With the above settings, no intrinsic PEEP was present under any experimental condition, as evidenced by the absence of tracheal pressure changes with airway occlusion at end-expiration..

*2.1 Procedure and data analysis*

Figure 1 provides a time line representation of the procedures performed. Eight animals (control group) were ventilated for 6-7 h on PEEP only, while sixteen animals (test

group) were subjected to the following sequence of PEEP and ZEEP: *a)* 1.5 h of mechanical ventilation (MV) with PEEP ($PEEP_1$); *b)* 3-4 h of MV at ZEEP; and *c)* 1.5 h of MV with PEEP ($PEEP_2$). The same end-expiratory pressure (2.3±0.1 $cmH_2O$) was applied during $PEEP_1$ and $PEEP_2$. Measurements were taken during $PEEP_1$ and $PEEP_2$, as well as at start ($ZEEP_1$) and end of the ZEEP period ($ZEEP_2$). In eight rabbits of the test group, measurements on $PEEP_1$ were done before and after 30 min from a single intravenous injection of the cyclo-oxygenase inhibitor acetylsalicylic acid (ASA; 15 $mg \cdot kg^{-1}$).

Two types of test breaths were used: *a)* while keeping $V_T$ at baseline values, $\dot{V}_I$ and $T_I$ were changed in the range 0.25 to 3 s to assess mechanics at end-inflation; and *b)* while keeping $\dot{V}_I$ constant, $T_I$ was changed to obtain the quasi-static inflation volume-pressure curve in the tidal volume range. End-inspiratory occlusions lasting 5 s were made in all test breaths, which were performed in random order and repeated 3-5 times. During ventilation with PEEP, the lungs were inflated 3-4 times to Ptr of ~25 $cmH_2O$ before all measurements, and the expiratory valve was opened to the ambient for 3-5 expirations in order to measure the difference between the end-expiratory and the resting lung volume (ΔEELV).

The quasi static elastance (Est), interrupter resistance (Rint), which reflects airway resistance, and viscoelastic resistance (Rvisc) and time constant (τvisc) were assessed according to the rapid airway occlusion method, as previously described (D'Angelo et al., 2002). The ratio ($R_{Est}$) between Est with the lowest inflation volume (~4 $ml \cdot kg^{-1}$) and baseline $V_T$ was taken as an index of peripheral airway recruitment-derecruitment during tidal ventilation (D'Angelo et al., 2007).

Tracheal NO concentration was continuously measured for 10-15 min at the beginning of MV on PEEP, at the transition from PEEP to ZEEP and from ZEEP to PEEP, and at ~60 and ~90 min after restoration of PEEP, to check for any possible deterioration of the preparation. For a given condition, 50 breaths were ensemble averaged, and the mean concentration of NO during expiration was used as the exhaled NO concentration (NOe). During ventilation on $PEEP_1$ and $PEEP_2$, part of the tubing beyond the expiratory valve of the ventilator was immersed in ice cold water for ~30 min to obtain on each occasion ~0.5 ml of exhaled airway vapor condensate (Hunt et al., 2000; Walsh et al., 2006). Assessment of $PGE_2$, $PGF_{2\alpha}$, and TNF-α concentration was performed on BALF and serum collected during the periods of NOe measurements.

Upon completion of *in vivo* measurements, the rabbits were given heparin (355 $U \cdot kg^{-1}$) and papaverin (5 $mg \cdot kg^{-1}$) intravenously to prevent bronchospasm, and killed with an overdose of anesthetics. The lungs and heart were removed *en block*. The right lung was

processed for histologic analysis as described below. The main left bronchus was tied off and cannulated, and the left lung was lavaged four times with a total of 4 $ml \cdot kg^{-1}$ of normal saline, care being taken to assess the amount of non-recovered fluid. The effluents were pooled, added with indomethacin to a final concentration of 10 $mg \cdot l^{-1}$, centrifuged (Harrier 18/80, Sanyo Gallenkamp PLC, Loughborough, UK) at 2000 rpm for 10 min, and the supernatant frozen and stored at –20°C. The lung was then weighed, left overnight in an oven at 120 °C, and weighed again to compute the wet/dry ratio, allowance being made for the non-recovered fluid. The animals were from a single cohort and the experiments were done in random order.

*2.2 Histologic analysis*

The pericardium was opened, the left branch of the pulmonary artery and left main bronchus were ligated, the left lung was removed, and a large needle inserted through the right ventricle into the pulmonary artery. After three inflations to 25 $cmH_2O$, the transpulmonary pressure was set at 10 $cmH_2O$ and the lung was perfused first with saline until the lobar surfaces became white, then with 4% formaldehyde, 0.1% glutaraldehyde dissolved in 0.12 M phosphate buffer, and finally kept immersed in 10% formaldehyde, 0.1% glutaraldehyde dissolved in 0.12 M phosphate buffer. Five blocks, ~1 cm thick, involving both subpleural and para-hilar regions, were obtained in each animal. From each block, processed through a graded series of alcohols and embedded in paraffin, sections of 5 μm thickness were cut and stained with hematoxylin-eosin.

Histologic evaluation was performed by a single observer in a blind fashion, according to the procedure previously described in details (D'Angelo et al., 2002; 2004). The following measures were obtained using a computer-aided, image analysis system (IMAQ Vision for LabView; National Instruments, Austin, TX): *a)* the percent ratio of injured (epithelial necrosis and sloughing) to total membranous bronchioles (IS), taken as a measure of small airway damage (Fig. 2); *b)* the percent ratio of abnormal to total (normal and abnormal) bronchiolar-alveolar attachments, taken as an index of airway-parenchymal mechanical uncoupling; and *c)* the number of polymorphonuclear leukocytes per unit length of alveolar septa, as an index of parenchymal inflammation. Parenchymal and vascular injury was further assessed by the presence of epithelial lesion of large (cartilaginous) airways, perivascular and peribronchial edema, focal alveolar collapse, and hemorrhage.

*2.3 Statistics.*

Results are presented as means±SE, except for histologic, prostaglandin, and TNF-α measurements which are expressed as median and range. For these measures the statistical analysis was performed using the Mann-Whitney test; otherwise the analysis of variance

(ANOVA) was used. When significant differences were found, the Bonferroni test was performed to determine significant differences between different experimental conditions. Linear regressions were computed with the least mean square method. The level for statistical significance was taken at $P \leq 0.05$.

## 3. Results

No significant differences occurred for any parameter between the control and test group at the beginning of the experiment. In eight rabbits of the test group, administration of ASA lowered serum $PGE_2$ and $PGF_{2\alpha}$ concentration significantly ($P<0.05$) from 85 to 42 $pg \cdot ml^{-1}$ and from 303 to 156 $pg \cdot ml^{-1}$, respectively, without affecting NOe concentration (-3.2±1.8 ppb; P=0.14). The behavior of NOe, serum TNF-α and prostaglandin concentration, lung mechanics, and $PaO_2$, $PaCO_2$, and pHa did not differ between untreated and ASA treated animals during the subsequent phases of the experiment: hence, data from all rabbits of the test group were pooled.

*3.1 NO production*

The effects of prolonged MV at PEEP or ZEEP on NOe concentration are shown in Figure 3, together with those on parameters that could potentially affect NOe. In the control group, NOe concentration was the same on $PEEP_1$ and $PEEP_2$. In the test group, NOe concentration increased significantly ($\Delta NOe=5\pm0.7$ ppb; $P<0.001$) on transition from PEEP to ZEEP, but was markedly decreased ($\Delta NOe=-17.9\pm1.8$ ppb; $P<0.001$) at the end of MV on ZEEP. No further significant changes in NOe concentration occurred during the subsequent 1.5 h of MV on PEEP, which was nearly half of the value on $PEEP_1$ (17.2±1.1 vs 33.2±2.4 ppb).

*3.2 Blood gasses and pH*

In the control group, $PaCO_2$, $PaO_2$, and pHa did not differ between $PEEP_1$ and $PEEP_2$ (Fig. 3). In the test group, there was a significant fall of $PaO_2$ on transition from PEEP to ZEEP. With prolonged MV on ZEEP, pHa decreased, $PaCO_2$ increased significantly, and $PaO_2$ showed a further fall. On transition from ZEEP to PEEP, $PaCO_2$ and eventually $PaO_2$ returned to the initial values, whereas pHa remained low. This was corrected with boluses of bicarbonate (1M) solution during the final measurements on $PEEP_2$. Systemic blood pressure decreased significantly on $ZEEP_2$, but returned to the initial values with restoration of physiological end-expiratory lung volumes, except in six animals in which this was obtained by means of epinephrine.

No significant differences were found between pH values of exhaled airway vapor condensate measured on $PEEP_1$ and $PEEP_2$ both in the control and test group (Fig. 3). When pH values measured in all animals and conditions were pooled, the average pH of the exhaled airway vapor condensate was 5.94±0.09 (range: 5.24-6.94).

*3.3 Prostaglandins and tumor necrosis factor*

The concentrations of $PGE_2$, $PGF_{2\alpha}$, and TNF-α in serum are shown in Figure 4. Prostaglandin concentration did not show any significant change throughout the experiment, both in the control and test group. When all data obtained on $PEEP_2$ were pooled, $PGE_2$ and $PGF_{2\alpha}$ concentration amounted to 174 $pg \cdot ml^{-1}$ (range: 0-1945) and 173 $pg \cdot ml^{-1}$ (range: 19-1023). As previously shown (D'Angelo et al., 2005; 2007), serum TNF-α concentration was high shortly after surgery (958 $pg \cdot ml^{-1}$; range: 100-12501) in both groups, but returned to essentially normal values during the initial period of ventilation on PEEP (21 $pg \cdot ml^{-1}$; range 0-900). No further changes in TNF-α concentration occurred on ZEEP and $PEEP_2$, when it amounted to 6 $pg \cdot ml^{-1}$ (range 0-197)

In BALF, $PGE_2$ and $PGF_{2\alpha}$ concentration were similar in both groups of rabbits, amounting to 139 $pg \cdot ml^{-1}$ (range: 0-408) and 245 $pg \cdot ml^{-1}$ (range: 26-749) in the control group, and 224 $pg \cdot ml^{-1}$ (range: 0-844) and 204 $pg \cdot ml^{-1}$ (range: 0-854) in the test group. Also the median value of TNF-α concentration was similar in the control and test group, amounting to 0 $pg \cdot ml^{-1}$ (range: 0-401) and 6 $pg \cdot ml^{-1}$ (range: 0-513), respectively.

*3.4 Lung mechanics*

As shown in Figure 5, no significant changes in quasi-static elastance (Est), interrupter (Rint) and viscoelastic resistance (Rvisc) and time constant (τvisc) occurred with prolonged MV in the control group. In the test group, Est, Rint and Rvisc increased relative to $PEEP_1$ by 88±9, 84±8, and 186±22% on $ZEEP_1$, and markedly more on $ZEEP_2$ (204±16, 233±30, and 320±33%), while τvisc did not change significantly. With restoration of the physiological end-expiratory lung volume ($PEEP_2$), Rvisc returned to the initial values, whereas Rint (45±6%; $P<0.001$) and Est (12±5%; $P<0.05$) remained elevated.

In the control group, the quasi-static inflation V-P curve and the resting lung volume (ΔEELV) were almost the same on $PEEP_1$ and $PEEP_2$. In the test group, ΔEELV was similar on $PEEP_1$ and $PEEP_2$, whereas the average slope of the quasi-static inflation V-P curve decreased slightly but significantly on $PEEP_2$ (Fig. 6). On ZEEP, this curve, which on PEEP was concave towards the pressure axis, shifted downwards and became s-shaped. As a consequence, the mean value of $R_{Est}$, which was significantly lower than unity and similar on

$PEEP_1$ and $PEEP_2$ in both groups of animals (0.88±0.03; $P<0.001$), became significantly larger than unity on ZEEP (Fig. 5).

*3.5 Histology*

No focal alveolar collapse or epithelial desquamation, alveolar, perivascular and peribronchial edema, damage of large airway epithelium, and hemorrhage was observed in either group of rabbits. Absence of edematous lesions was further confirmed by the mean values of lung wet-to-dry ratio in the control and test group (4.7±0.1 and 4.9±0.2, respectively) being not significantly different from 4.6±0.1 of freshly excised, normal rabbit lungs (D'Angelo et al., 2002), as well as by the very low ratio of BALF to serum albumin concentration (1.3±0.4 and 0.9±0.1%, respectively).

Indices of peribronchiolar alveolar wall destruction (% abnormal attachments), bronchiolar epithelial injury (IS), and trapping of granulocytes in the alveolar wall (cells) are shown in Table 1. All parameters were significantly higher in the test than control group.

## 4. Discussion

In line with previous observations (D'Angelo et al., 2005), prolonged mechanical ventilation with physiological end-expiratory and tidal volume in normal, open chest rabbits has no effects on NO concentration of exhaled air, serum TNF-α concentration, and lung mechanics (Fig. 3-6). Moreover, it did not affect serum prostaglandin concentration. In contrast, prolonged mechanical ventilation with physiological tidal volumes and low end-expiratory volumes in normal, open chest rabbits causes a marked reduction of NO concentration in exhaled air (Fig. 3) and mechanical alterations (Fig. 5 and 6) that persist after restoration of physiological end-expiratory lung volumes, with concurrent generation of histologic injury of small airways (Table 1). The present results have shown that among the several factors potentially responsible for this decrease in NOe, the more likely cause is the damage of small airway epithelium induced by the abnormal stresses that develop with cyclic airway opening and closing, hereafter referred to as tidal airway closure.

The initial NOe concentration in the open chest rabbits was comparable with that found in intact, normal anesthetized rabbits (D'Angelo et al., 2005; Forsberg et al., 1999). On transition from PEEP to ZEEP there was a significant immediate increase in NOe concentration (Fig. 3), likely due to the decrease of the alveolar-capillary surface and pulmonary blood flow and volume that occurs with decreasing lung volume, thus leading to reduced NO uptake by the pulmonary circulation (Carlin et al., 1997). However, at the end of the period of mechanical ventilation on ZEEP, NOe levels were decreased to ~45% of the initial values, with a concomitant significant increase in $PaCO_2$ and decrease of $PaO_2$ and pHa

(Fig. 3). Both hypoxia and hypercapnia reduce NOe concentration (Agvald et al., 2005; Carlin et al., 1997; Cremona et al., 1995), while the effects of acidosis are controversial (Agvald et al., 2005; Pedoto et al., 1999). Although these factors might have contributed to the reduced NOe concentration on ZEEP, they were not responsible for its persistent fall on $PEEP_2$, because NOe levels remained well below control values long after return to normal end-expiratory volume and initial $Pa_{O_2}$, $Pa_{CO_2}$, and pHa values (Fig. 3). Moreover, the fall of NOe on $PEEP_2$ could not have been due to changes in the pattern of regional lung emptying, as it should have been essentially the same on $PEEP_1$ and $PEEP_2$. In fact, no appreciable changes occurred between these two conditions in the standard time constant and tissue viscoelastic parameters (Fig. 5), the latter also accounting for the inequality of parallel time constants, the operating lung volumes (Fig. 6), the breath timing, and eventually the expiratory flow rate. Furthermore, inhomogeneous ventilation did not affect NOe appreciably in the present rabbits: in fact, there was no change in NOe concentration from ZEEP2 to PEEP2 (Fig.3), whereas inhomogeneous lung expansion should have occurred in the former but not in the latter condition (Fig. 5 and 6; see below). Finally, intravenous infusions of epinephrine has been shown to cause a dose dependent increase of NOe in anesthetized and mechanically ventilated rabbits (Adding et al., 1999). Nevertheless, single epinephrine injections performed on $PEEP_2$ in a few animals of the test group to restore normal blood pressure had no appreciable effects on NOe concentration, probably because of the small cumulative dose.

Changes in the volume or composition of the fluid lining the lower airways can lower NOe, as shown in isolated pig lungs with developing interstitial edema (Cremona et al., 1995), in healthy subjects with administration of nebulized aqueous solutions (Maniscalco et al., 2001), and in normal subjects and asthmatic patients with inhalation of buffer solution (Gaston et al., 2006), whereas the concomitant increase in hydrogen ions concentration of the lining fluid and exhaled vapor condensate is paralleled by an increased NOe (Hunt et al., 2000; Kostikas et al., 2002; Walsh et al., 2006). However, no appreciable interstitial edema occurred in the present animals, as shown by normal wet-to-dry ratio and albumin concentration in BALF and absence of perivascular and peribronchial cuffs, while the pH of the condensate remained almost constant (Fig. 3), thus making it unlikely that the NOe changes were due to factors operating downstream from NO synthase (Gaston et al., 2006, Snyder et al., 2002).

Prostaglandin $E_2$ and $F_{2\alpha}$ can exert a potent depressant effect on NO production (Delgado et al., 1999; Kharitonov et al., 1999). Damage and shedding of airway epithelia, by exposing nerve endings, fibroblasts, and collagen, can eventually cause activation of bradykinin with release of $PGE_2$ and $PGF_{2\alpha}$, while an additional source could be the alveolar

epithelial cells and granulocytes activated by mechanical insults (Delgado et al., 1999). Prolonged mechanical ventilation at low lung volume did in fact cause necrosis and sloughing of small airway epithelium and increased number of granulocytes in the alveolar walls of the test group (Table 1). Nevertheless, serum $PGE_2$ and $PGF_{2\alpha}$ levels remained essentially the same throughout the whole experimental procedure (Fig. 4), and their concentration in BALF were similar in both groups, suggesting that $PGE_2$ and $PGF_{2\alpha}$ are not involved in the decrease of NOe. This is further supported by the absence of compartmentalization of $PGE_2$ and $PGF_{2\alpha}$ production, since in the test group *a)* no difference in the decrease of NOe concentration with ZEEP ventilation was found between untreated and ASA treated animals, although ASA effectively reduced serum $PGE_2$ and $PGF_{2\alpha}$ levels; and *b)* no significant relationship could be demonstrated between individual values of $PGE_2$ and $PGF_{2\alpha}$ concentration in BALF and corresponding changes in NOe levels from the initial measurements on $PEEP_1$ to the final ones on $PEEP_2$ (Fig. 7). On the other hand, the increased number of alveolar granulocytes in the lungs of the test relative to the control group does not seem to represent a sign of inflammation, as both TNF-α and prostaglandin levels in serum and BALF were normal. Indeed, such an increase occurred also after prolonged ventilation on ZEEP in animals treated with exogenous surfactant, in which no mechanical and histological damage developed (D'Angelo et al, 2007). Hence, the fall in NOe concentration with mechanical ventilation at low lung volumes occurs in the absence of inflammatory response.

Surfactant dysfunction with reduced lung volume should cause airway closure, gas trapping, microatelectasis, and reduction in ventilated tissue, leading to a progressive increase of quasi-static elastance and airway resistance (Fig. 5), and tidal airway closure, as shown by the change in shape of the quasi-static P-V curve in the tidal volume range (Fig. 6) and the marked increase (~40%) of $R_{Est}$ from PEEP to ZEEP (Fig. 4). Inhomogeneous lung expansion with tidal airway closure in the presence of increased surface tension implies the generation of abnormal stresses, which are eventually responsible for the histologic damage of small airway epithelium and bronchiolar-alveolar attachments (Table 1), marked alterations of alveolar-bronchiolar coupling and increase in airway resistance and quasi-static lung elastance, that persists after restoration of physiological end-expiratory volumes (Fig. 5). This sequence of events is supported by the observation that in normal open chest rabbits, administration of exogenous surfactant largely prevents both the histologic and functional alterations of mechanical ventilation at low lung volumes (D'Angelo et al., 2007).

In animals of the test group, necrosis and epithelial sloughing involved ~25% of peripheral airways, an estimate that compares well with the average bronchiolar injury score obtained from previous studies, damage which was essentially absent in animals ventilated

with physiological end-expiratory lung volumes (Table 1). Actually, the extension of epithelial injury might have been even greater, as no allowance could be made of the possible, partially reversible plasma membrane stress failure (Gajic et al., 2003). The final fall in NOe concentration averaged 45%, which is similar to the 35% decrease found in 16 open and closed chest rabbits, in which, however, no histologic observations had been made (D'Angelo et al., 2005). Considering that other factors potentially capable to cause a fall in NOe were not operating (see above), the satisfactory correspondence between percentage of injured airways and relative decrease of NOe could suggest that the latter is due to the mechanically induced damage of small airway epithelium with consequent fall in NO production. This is supported by the significant correlation found in the test group between individual values of bronchiolar injury score and corresponding changes in NOe levels from the initial to the final ones on PEEP (Fig. 7), a correlation which was absent in the control group, and is consistent with the conclusion from model analysis in humans indicating that during normal breathing and exclusion of upper airway contribution, most of NOe is contributed by airway epithelium, especially small airways (Condorelli et al, 2006; Shin and George, 2002; Van Muylem et al., 2003). Moreover, airway epithelial injury can reduce NOe also by increasing the NO transfer factor of the airway compartment.

The present results could be relevant to those circumstances in which the closing volume exceeds the end-expiratory volume and tidal airway closure ensues, because they would predict a decreased NOe concentration in all conditions characterized by tidal airway closure, such as morbid obesity, COPD, ARDS-ALI, and chronic heart failure (Milic-Emili et al., 2007). Indeed, this is the case in chronic heart failure (Bussotti et al., 2004; Lovell et al., 2000) and severe obesity (Maniscalco et al., 2007). In the other conditions and in the presence of an inflammatory reaction, NOe concentration could, however, result from the balance of factors that increase (e.g. cytokines) and depress (e.g. tidal airway closure) the NO elimination from peripheral airways. This, together with the possible contribution of the concomitant stress induced damage of the pulmonary endothelium, would explain the variable behavior of NOe concentration that has been frequently observed in patients with COPD (Ansarin et al., 2001; Brindicci et al, 2005; Clini et al., 1998; Rutgers et al., 1999) and ARDS (Brett and Evans, 1998; Gessner et al., 2003). Interestingly, in patients with acute lung injury higher urinary NO levels and improved outcomes have been observed during mechanical ventilation with low rather than high tidal volume (McClintock et al., 2007), suggesting that higher endogenous NO could reflect a better preserved response to pro-inflammatory mediators of airway epithelium and pulmonary endothelium, as a result of reduced peripheral airway involvement in tidal airway closure with smaller tidal volumes and hence less stress-induced injury.

In conclusion, the present results have shown that prolonged mechanical ventilation at low lung volumes in normal, open chest rabbits causes a marked reduction of NO concentration in exhaled air and mechanical alterations, which persist after restoration of physiological end-expiratory lung volumes, with concurrent generation of histologic injury of peripheral airways. The fall of NOe occurs in the absence of inflammatory reaction and is most likely attributable to epithelial damage of the small airways due to abnormal shear stress that develop with tidal airway closure. This could support the practical use of NOe as an indication of peripheral airway epithelial injury.

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Table 1. Indices of lung injury in open chest rabbits subjected to prolonged mechanical ventilation with physiological (control group) or low end-expiratory volumes (test group)

| | Cells $mm^{-1}$ | A-A % | IS % | N |
|---|---|---|---|---|
| *Control group* | 0.3 (0.1-0.3) | 9 (4-19) | 9 (3-15) | 8 |
| *Test group* | 1.0* (0.4-2.5) | 26* (21-33) | 25* (13-44) | 16 |

Values are medians with range in parentheses. Cells, number of polymorphonuclear leukocytes per unit length of alveolar septa; A-A, percentage of ruptured bronchiolar-alveolar attachments; IS, percentage of membranous and respiratory bronchioles with epithelial lesions; N, number of animals. *Significantly different from corresponding values of control group ($P<0.01$).

Legends

Fig. 1 Time line representation of the procedure used in eight rabbits ventilated only with physiological end-expiratory volume (control group) and sixteen rabbits ventilated also with low end-expiratory volume (test group). NOe, TNF-α, $PGE_2$, $PGF_{2\alpha}$, $PaO_2$, $PaCO_2$, and pHa are exhaled nitric oxide, serum tumor necrosis factor and prostaglandin $E_2$ and $F_{2\alpha}$, arterial partial pressure of oxygen and carbon dioxide, and pH, respectively. Dot indicate NOe assessment followed by acetylsalicylic acid administration performed in eight rabbits of the test group. PEEP and ZEEP are positive and zero end-expiratory pressure, respectively, while subscripts 1 and 2 refer to periods of PEEP and ZEEP.

Fig. 2. Microphotographs showing a bronchiole with (arrows) and one without epithelial lesions. Dotted arrows indicate abnormal bronchiolar-alveolar attachments. See also in D'Angelo et al. (2004). Calibration bar is 100 μm.

Fig. 3. Average values of concentration of nitric oxide in expired air (NOe), pH of deareated, exhaled airway vapor condensate (pH cond), arterial pH (pHa), partial pressure of carbon dioxide ($PaCO_2$) and oxygen ($PaO_2$), and mean arterial pressure ($\overline{Pa}$) in 8 open chest rabbits ventilated with a PEEP of 2.3 $cmH_2O$ (control group) and in 16 open chest rabbits (test group) during ventilation with the same PEEP before ($PEEP_1$) and after 3-4 h of ventilation on ZEEP ($PEEP_2$), and during the initial ($ZEEP_1$) and final period ($ZEEP_2$) of ventilation on ZEEP. Bars: SE. Values significantly different from corresponding ones on $PEEP_1$: *P<0.05; **P<0.01. Number in parentheses indicate the average time (minutes) elapsed from the completion of the surgical procedure.

Fig. 4. Box plot of serum prostaglandin ($PGE_2$ and $PGF_{2\alpha}$) and tumor necrosis factor (TNF-α) concentration in 8 open chest rabbits ventilated with a PEEP of 2.3 $cmH_2O$ (control group) and in 16 open chest rabbits (test group) during ventilation with PEEP of 2.3 $cmH_2O$ before ($PEEP_1$) and after 3-4 h of ventilation on ZEEP ($PEEP_2$), and during the final period ($ZEEP_2$) of ventilation on ZEEP. Number in parentheses indicate the average time (minutes) elapsed from the initial measurements on PEEP.

Fig. 5. Mean values of quasi-static elastance (Est), interrupter (airway) resistance (Rint), tissue viscoelastic resistance (Rvisc) and time constant (τvisc), and ratio between Est measured with the lowest and baseline $V_T$ ($R_{Est}$) obtained in 8 open chest rabbits ventilated with a PEEP of 2.3 $cmH_2O$ (control group) and in 16 open chest rabbits (test group) during ventilation with PEEP before ($PEEP_1$) and after 3-4 h of ventilation on ZEEP ($PEEP_2$), and at the beginning ($ZEEP_1$) and end of the 3-4 h period ($ZEEP_2$) of ventilation on ZEEP. Bars: SE. Values significantly different from corresponding ones on $PEEP_1$: *P<0.05; **P<0.01.

Fig. 6. Average relationships obtained in the tidal volume range between volume above resting lung volume (ΔV) and quasi-static transpulmonary pressure in 16 open chest rabbits (test group) during ventilation with PEEP of 2.3 $cmH_2O$ before ($PEEP_1$) and after 3-4 h of ventilation on ZEEP ($PEEP_2$), and during the initial ($ZEEP_1$) and final period ($ZEEP_2$) of ventilation on ZEEP (see key to symbols). Bars: SE.

Fig. 7. The relationship between relative changes in exhaled nitric oxide concentration (NOe) from the beginning to the end of the experiment and prostaglandin $E_2$ and $F_{2a}$ concentration in bronchoalveolar lavage fluid (*upper panel*) or injury score (*lower panel*) computed as the percent ratio of damaged to total respiratory and membranous bronchioles obtained in 16 open chest rabbits (test group) ventilated at low end-expiratory lung volume.

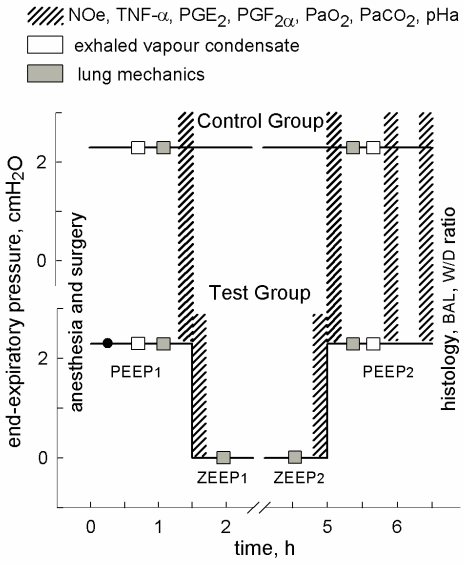
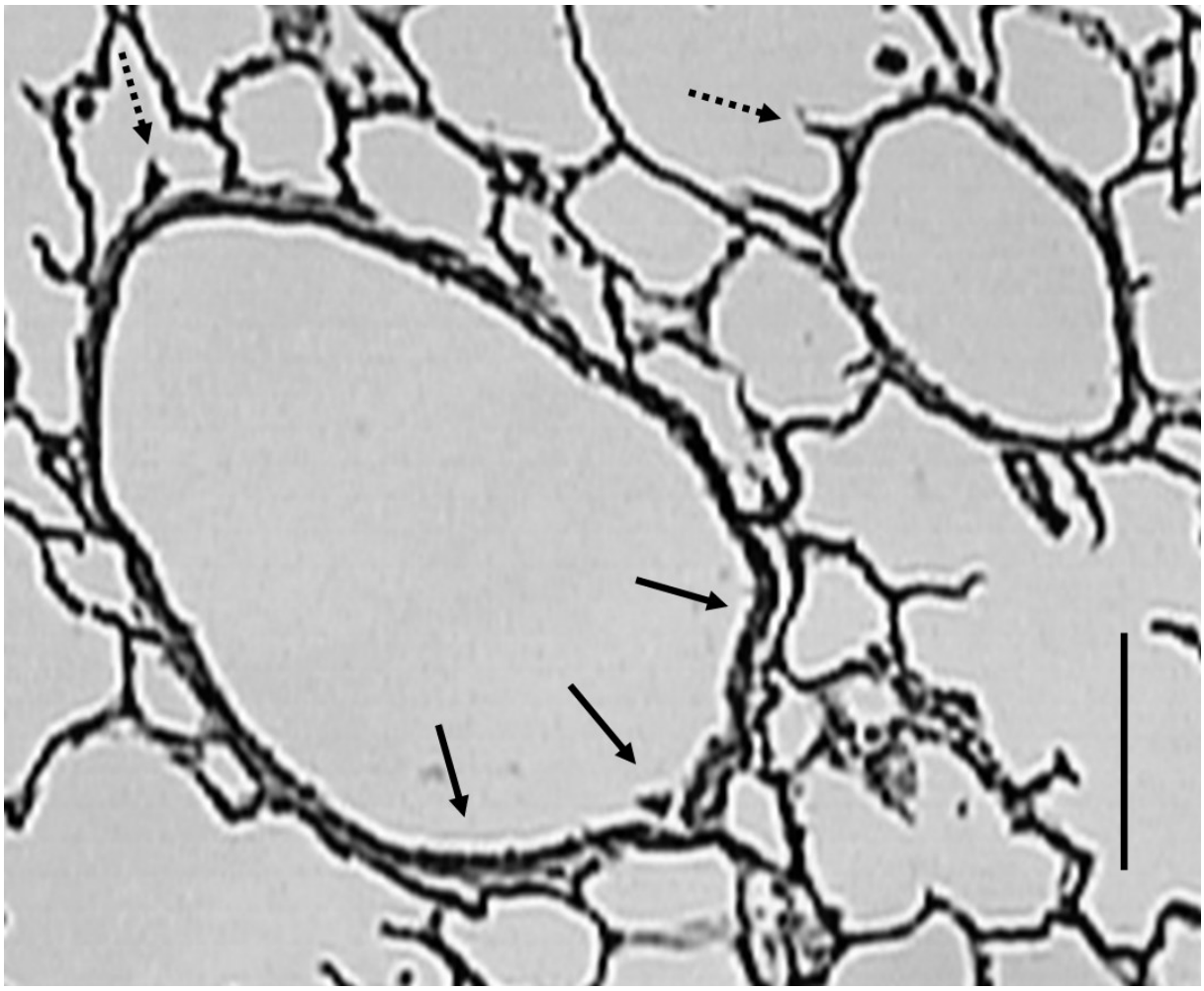

NOe, TNF-α, PGE2, PGF2α, PaO2, PaCO2, pHa
exhaled vapour condensate
lung mechanics
Control Group
Test Group
end-expiratory pressure, cmH2O
2
0
2
0
anesthesia and surgery
PEEP1
ZEEP1
ZEEP2
PEEP2
histology, BAL, W/D ratio
0
1
2
5
6
time, h

Figure 1



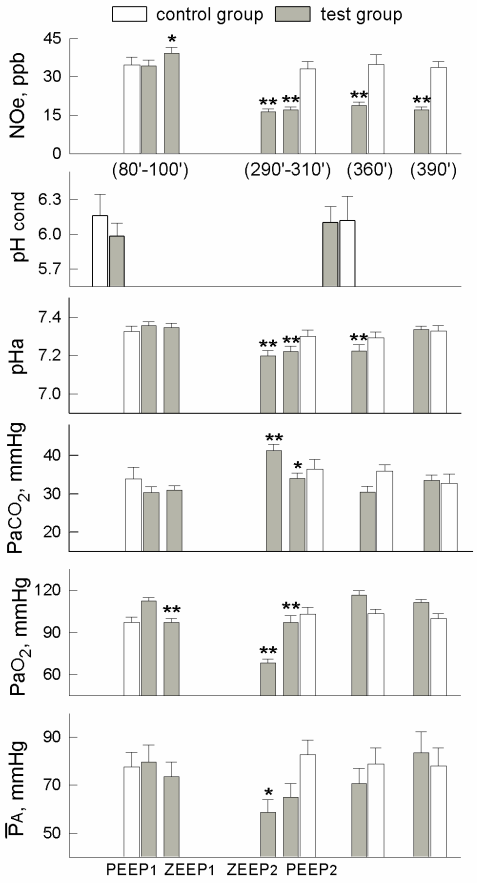

control group
test group
NOe, ppb
45
30
15
0
(80'-100')
(290'-310')
(360')
(390')
pH cond
6.3
6.0
5.7
pHa
7.4
7.2
7.0
PaCO2, mmHg
40
30
20
PaO2, mmHg
120
90
60
P̄A, mmHg
90
70
50
PEEP1 ZEEP1 ZEEP2 PEEP2

Figure 3

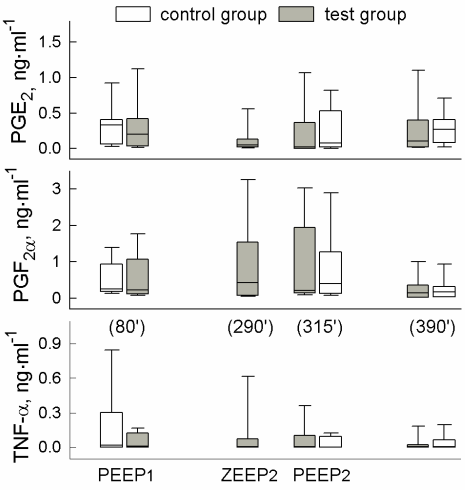

control group
test group
PGE2, ng·ml-1
1.5
1.0
0.5
0.0
PGF2α, ng·ml-1
3
2
1
0
(80')
(290')
(315')
(390')
TNF-α, ng·ml-1
0.9
0.6
0.3
0.0
PEEP1
ZEEP2
PEEP2

Figure 4

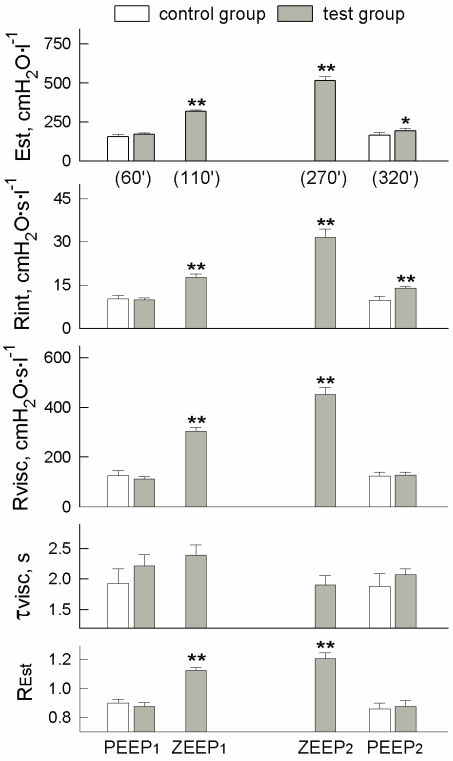

control group
test group
Est, cmH2O·l-1
750
500
250
0
**
**
*
(60')
(110')
(270')
(320')
Rint, cmH2O·s·l-1
45
30
15
0
**
**
**
Rvisc, cmH2O·s·l-1
600
400
200
0
**
**
τvisc, s
2.5
2.0
1.5
REst
1.2
1.0
0.8
**
**
PEEP1
ZEEP1
ZEEP2
PEEP2

Figure 5

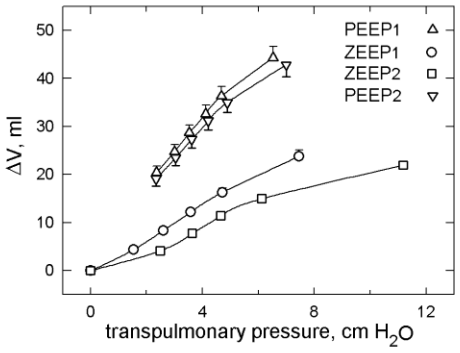

PEEP1
ZEEP1
ZEEP2
PEEP2
ΔV, ml
50
40
30
20
10
0
0
4
8
12
transpulmonary pressure, cm H2O

Figure 6

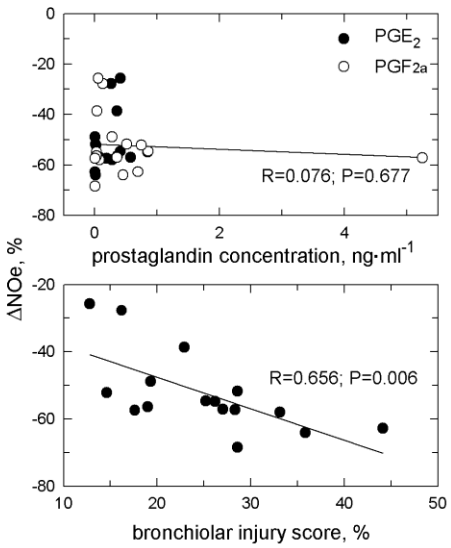

PGE2
PGF2a
R=0.076; P=0.677
prostaglandin concentration, ng·ml-1
ΔNOe, %
R=0.656; P=0.006
bronchiolar injury score, %

Figure 7