Rat models of acute lung injury: Exhaled nitric oxide as a sensitive, noninvasive real-time biomarker of prognosis and efficacy of intervention

Fangfang Liu\textsuperscript{a,c,1}, Wenli Li\textsuperscript{b,1}, Jürgen Pauluhn\textsuperscript{c,*}, Hubert Trübel\textsuperscript{d}, Chen Wang\textsuperscript{c,**}

\textsuperscript{a} Beijing Institute of Respiratory Medicine, Capital Medical University, Beijing 100069, China
\textsuperscript{b} Department of Toxicology, Fourth Military Medical University, Xi'an 710032, China
\textsuperscript{c} Department of Toxicology, Bayer Pharma AG, 42096 Wuppertal, Germany
\textsuperscript{d} Department of Pharmacology Vascular Diseases, Cardiology & Hematology, Bayer Pharma AG, 42096 Wuppertal, Germany
\textsuperscript{*} Beijing Institute of Respiratory Medicine, Beijing Hospital, Ministry of Health, Beijing 100730, China

\begin{abstract}
Exhaled nitric oxide (eNO) has received increased attention in clinical settings because this technique is easy to use with instant readout. However, despite the simplicity of eNO in humans, this endpoint has not frequently been used in experimental rat models of septic (endotoxemia) or irritant acute lung injury (ALI). The focus of this study is to adapt this method to rats for studying ALI-related lung disease and whether it can serve as an instant, non-invasive biomarker of ALI to study lung toxicity and pharmacological efficacy. Measurements were made in a dynamic flow of sheath air containing the exhaled breath from spontaneously breathing, conscious rats placed into a head-out volume plethysmograph. The quantity of eNO in exhaled breath was adjusted (normalized) to the physiological variables (breathing frequency, concentration of exhaled carbon dioxide) mirroring pulmonary perfusion and ventilation. eNO was examined on the instillation/inhalation exposure day and first post-exposure day in Wistar rats intratracheally instilled with lipopolysaccharide (LPS) or single inhalation exposure to chlorine or phosgene gas. eNO was also examined in a Brown Norway rat asthma model using the asthmagen toluene diisocyanate (TDI). The diagnostic sensitivity of adjusted eNO was superior to the measurements not accounting for the normalization of physiological variables. In all bioassays – whether septic, airway or alveolar irritant or allergic, the adjusted eNO was significantly increased when compared to the concurrent control. The maximum increase of the adjusted eNO occurred following exposure to the airway irritant chlorine. The specificity of adjustment was experimentally verified by decreased eNO following inhalation dosing of the non-selective nitric oxide synthase inhibitor amoniguanidine. In summary, the diagnostic sensitivity of eNO can readily be applied to spontaneously breathing, conscious rats without any intervention or anesthesia. Measurements are definitely improved by accounting for the disease-related changes in exhaled CO\textsubscript{2} and breathing frequency. Accordingly, adjusted eNO appears to be a promising methodological improvement for utilizing eNO in inhalation toxicology and pharmacological disease models with fewer animals.
\end{abstract}

\section{1. Introduction}
Nitric oxide (NO) is a highly diffusible gas and can be detected in human breath as an instant, non-invasive biomarker of different types of lung diseases and certain inflammatory airway disorders. NO is a free radical and biologically active autacoid which is synthesized by the action of NO synthase (NOS) in a number of cells and especially the endothelial cells of the pulmonary capillaries. It is a key physiological mediator of pulmonary function, and plays a critical role in the lung vascular and airway tone regulation. Excessive NO synthesis is attributed to the inducible NOS (iNOS); however, any imbalance of iNOS with its constitutive isoforms, the endothelial NOS and neuronal NOS, has also been implicated in the pathophysiology of many cardiopulmonary diseases (Vallance and Leiper, 2002; Vaughan et al., 2003). The over-shooting production of NO is conducive to the formation of peroxynitrite, a highly reactive mediator of tissue destruction, inflammation and...
vasoconstriction (Beckman and Kpenol, 1996; Hesslinger et al., 2009). The expression of these isofoms is highly localized and tissue-specific with different and sometimes opposing pharmacodynamic effects. The outcome of pharmacological intervention by NOS inhibitors is depending on the tissue-specific dose, route, and its selectivity (Hauser et al., 2005; Hesslinger et al., 2009).

The analysis of biomarkers in exhaled breath has widely been used in respiratory research settings (Kharitonov and Barnes, 2001a,b), among which the exhaled nitric oxide (eNO) is the most extensively studied marker. Measurement of eNO is a direct, simple, non-invasive and valid tool to estimate NO production in pathological conditions such as endotoxemia, acute lung injury (ALI) and airway inflammation (Bachetti et al., 2003; Bernareggi et al., 1999; Birrell et al., 2006; Condorelli et al., 2007; Ignarro, 1989; Lundberg et al., 1996; Stitt et al., 1997; Ricciardolo et al., 2004). Increased levels of eNO have been reported in asthma and chronic obstructive pulmonary disease (COPD) (Alving et al., 1993; Brindici et al., 2005).

In asthmatic patients, eNO levels have been shown to correlate with sputum eosinophilia, eosinophilia in bronchoalveolar lavage fluid (BALF), and peak-flow variability (Gibson et al., 2000; Mattes et al., 1999; Jatakanon et al., 1998; Lim et al., 1999; Berry et al., 2005). Thus, NO is thought to be an important modulator of airway function in normal and inflamed airways and eNO is regarded as a relatively reliable marker reflecting airway injury and response to treatment.

The understanding of the origins of the eNO from the lung and its relationship to pulmonary surface area and circulation are important factors in regard to the diagnostic utility of this biomarker. Due to the high diffusivity of NO one might speculate that the NO produced locally within the highly perfused endothelium of the alveolar capillaries may only be partially discharged into the alveoli from the circulation. Stitt et al. (1997) conducted experiments in rats to evaluate the possibility that the pulmonary blood flow acts as a sink to any NO that is presented by the lung. These authors conclude that the pulmonary blood flow more likely removes NO from the lung rather than delivering it into the pulmonary airways. However, at pathological stages with dysfunctional air–blood barrier, a more protracted back-diffusion into the vascular system can be anticipated. On the other hand, the NO produced intraluminally by inflammatory cells may either re-equilibrate at the blood-air barrier or is exhaled. At locations distal to the gas exchange region, diffusional processes are less efficacious due to increased tissue thickness, smaller surface area, and decreased perfusion. From that one may deduce that the diagnostic sensitivity of this biomarker can be improved by accounting for ventilation–perfusion-related variables of eNO.

These variables appear to be particularly important when using this assay in conscious, spontaneously breathing rats. Each type of method used to sample eNO and disease-specific changes in tidal breathing and hemodynamics (lung perfusion) may affect the measured concentration of NO in exhaled breath. A variety of eNO measurement methods have been reported in previous studies (Mehta et al., 1997; Stitt et al., 1997; Zegdi et al., 2003; Nials et al., 2011). However, most of these diverse methods are difficult to implement in inhalation toxicity studies on rats as they require extensive instrumentation, additional interventions and anesthesia or terminal tracheostomy.

The purpose of the present study was to systematically analyze and compare three different methods of non-invasive, repeated eNO-collection in unanesthetized or superficially anesthetized rats without any surgical intervention. Following this comparison of different methods in lipopolysaccharide (LPS) treated rats (septic ALI), the most sensitive and expedient method was then chosen to compare eNO measurements from four different types of lung inflammation: (1) acute septic inflammation by LPS instillation, (2) acute alveolar inflammation by phosgene gas inhalation, (3) acute airway inflammation by chlorine gas inhalation, and (4) allergic inflammation by tolueine diisocyanate (TDI) vapor provocation of previously sensitized rats. This included a comparative analysis whether the diagnostic sensitivity of eNO can be improved by an adjustment that accounts for the disease-specific differences in ventilation and lung perfusion relative to normal control rats. The specificity of this adjustment was verified by comparing phosgene-exposed rats with versus without additional post-exposure treatment with the iNOS-inhibitor aminoguanidine.

2. Methods

2.1. Test material

Lipopolysaccharides (LPS), from Escherichia coli 055:B5; tolueine diisocyanate (TDI), and aminoguanidine (aminoguanidine hemisulfate salt) were from Sigma, Germany. Chlorine (certified gas of 400 ppm in synthetic air, cylinder) and phosgene (carbonyl chloride, certified gas of 150 ppm in synthetic air, cylinder) were from Linde, Germany.

2.2. Animals, diet, and housing conditions

Healthy male SPF-bred Wistar rats of the strain Hsd Cpb:WU from the experimental animal breeder Harlan-Nederland (NL), AD Horst, and male Brown Norway rats of the strain BN/CRi BR purchased from Charles River, Sulzfeld, Germany, were used. Animals were placed in polycarbonate cages containing bedding material. Both feed and water were given ad libitum except during inhalation exposures. At the commencement of study, the mean body weights of Wistar rats were approximately 210–240 g, and that of Brown Norway rats were approximately 220–250 g. Animal rooms were maintained at approximately 22 °C with relative humidity of 40–60% and a 12-h light cycle beginning at 0600 h. The studies described were in accordance with contemporary, internationally harmonized testing standards/guidelines (OECD, 2009, available at: http://oberon.sourceforge.net). The experiments were performed in an animal care–approved laboratory in accordance with the German Animal Welfare Act and European Council Directive 86/609/EEC (Directive 86/609/EEC, 1986) as well the updated Directive 2010/63/EU as of 22 September 2010.

2.3. Experimental procedures

Three different methods of collection of eNO were systematically evaluated and compared in LPS treated rats (septic ALI). Method-1 utilized conscious, spontaneously breathing rats in a head-out (gas exchange compartment) volume displacement plethysmograph (Fig. 1). Further details of this method are described below “Measurements of Nitric Oxide, Carbon Dioxide, and Breathing Frequency”.

Method-2 utilized anesthetized rats which were mechanically ventilated with synthetic air via an orotracheal cannula connected to a ventilator. The exhaled air from the ventilator (7025 Rodent Ventilator, Ugo Basile, tidal volume: 2 mL, frequency: 75 strokes/min) was collected in a gas sampling bag (Cali-5-BondTM, Ritter, Germany; volume: 2.0 L). The advantages of this method overcome the shortcomings of methods-1 and method-3 because the exhaled volume can be exactly quantified and a carrier air-flow is not required. Rats were anesthetized with isoflurane (initially 4% with reduction after attainment of anesthesia). For analysis the gas filled bag was connected directly to the NO analyzer for NO analysis. Method-3 was similar to method-1; however, in using whole-body barometric bias-flow plethysmograph (volume: 1.8 L; bias flow-rate 0.75 L/min) instead of a volume displacement plethysmograph.

Based on the outcome of the LPS study, the least invasive, most sensitive and expedient means to measure eNO was achieved by method-1. Therefore, only this method was used to study eNO in three other types of phosgene ALI (alveolar irritation), chlorine ALI (airway irritation) and allergic ALI (tolueine diisocyanate, TDI vapor provocation of previously sensitized rats). Particular emphasis was directed toward the question whether any additional adjustment of eNO for the disease-specific differences in ventilation (measurement of breathing frequency) and lung perfusion (approximated by measurement of CO2 in exhaled breath) improves the sensitivity of this assay. The specificity of this adjustment was exemplified by comparing phosgene-exposed rats with/without additional post-exposure treatment with the iNOS-inhibitor aminoguanidine.

- Septic ALI: Each comparison utilized three Wistar rats/group receiving either LPS or the vehicle saline under otherwise identical conditions. Method validation utilized LPS (5 mg/kg body weight, 1 ml/kg body weight, intratracheal instillation) to produce a septic ALI. eNO was measured on days 0 (1 and 6h post-IT) and 1 (approximately 24h post-IT). The IT dosing procedure and the associated time-course change in ALI progression have been published in detail previously (Liu et al., 2013).
**Irritant ALI:** Wistar rats were directed-flow nose-only exposed by inhalation for 30 min. Each gas was metered from the cylinder into the inhalation chamber under highly controlled conditions. The integrity and stability of the vapor generation and exposure system was measured real-time by using the calibrated Gasmet DX-4000 FT-IR. Samples were taken continuously from the vicinity of the rats ’ breathing zone. The validation of this inhalation system has been published elsewhere (Pauluhn, 2006a,b; Pauluhn and Thielt, 2007). The airway-ALI was induced by the exposure to 12,000 ppm × min chlorine (400 ppm chlorine for 30 min). This concentration was selected based on published evidence (Lesuk et al., 2008; Yadav et al., 2010). The alveolar-ALI rats were similarly exposed to 1050 mg phosgene/m³ × min (≈35 mg phosgene/m³ for 30 min). This concentration has been used in previous studies (for details see Li et al., 2011, 2013; Pauluhn, 2006a,b). Three rats/group were examined for eNOS 5 and 24 h post-exposure. Each series of measurements required approximately 90 min. Control rats were exposed to conditioned air under otherwise identical conditions. Rectal temperatures were measured using a rectal probe for rats & mice (flexible thermo-probe; Hugo Sachs Elektronik-Havard Apparatus, Scientific Instruments, March/Freiburg, Germany) shortly before measurements.

**Aminoguanidine inhalation:** Aminoguanidine (dissolved 7 and 23% (w/v) in deionized water at 300 and 1000 mg/m³) was aerosolized using a modified BGI 3-nozzle Collision nebulizer (Type CN-25 MRE, BGI Inc., Waltham MA, USA). Rats were directed-flow nose-only exposed twice for 30 min to aminoguanidine aerosol, starting at 0.5 and 4 h after exposure to phosgene (for more details see Li et al., 2011, 2013).

**Allergic airway disease:** Brown Norway rats were sensitized topically (contralateral flanks) on days 0 and 7 to TDI or vehicle. For the elicitation of respiratory allergy the rats were challenged 3-times by inhalation for a duration of 30-min at a target concentrations of 80 mg TDI/m³ on target days 21, 35, and 49. At the last challenge (target day 63), the rats of the equally challenged control and TDI-induction groups were subjected to a stepped challenge (8 rats/group) at 15 and 45 mg TDI/m³ under otherwise identical conditions (2–3 h post-challenge, 4 rats per sub-group). The validation of this Brown Norway Asthma model has been detailed elsewhere (Pauluhn, 2008; Pauluhn and Poole, 2011).

### 2.4. Measurements of nitric oxide, carbon dioxide, and breathing frequency

The time points for eNOS analysis were selected based on the rationale to detect the earliest point of departure from normal and a second time point in the range of the maximum pulmonary response to acute lung injury. Based on previous studies, these time points were met best by measurements approximately 5 and 24 h post-instillation/exposure to the ALI-inducing agent (Liu et al., 2013; Li et al., 2013). For detection of the allergic inflammation measurements 2–3 h post-provocation were considered appropriate (Pauluhn and Poole, 2011).

**Nitric Oxide in exhaled breath:** NO was analyzed real-time using a chemiluminescence analyzer (Sievers 280B NOA; Sievers Instrument, Inc., Denver, CO). Synthetic air was used during measurements of exhaled NO. The actual concentration of exhaled NO (eNO) was not accounted for the residual background level of NO which was approximately 15 ppb. Measurements were preferentially made in two-compartment head-out volume displacement plethysmographs (in-house design; method-1) based on the rationale given below. Data were recorded sequentially every 10 s for 10 min at specified post-exposure/instillation time points. The ‘head-compartment’ of the plethysmograph (volume: ≈95 mL) was dynamically flushed with synthetic air (0.75L/min). This flow rate is about 3-times higher than the respiratory minute volume of the rats used (≈0.25L/min-rat; Pauluhn and Thielt, 2007) and 10-times higher than the total dead-space volume (head-displacement volume of rat subtracted). During the exposure period air flows were monitored and controlled continuously by calibrated mass-flow meters (see Fig. 1). The total exhaust flow was controlled by a vacuum pump (Edwards RV3). For the calibration of air-flow devices a DryCal Defender 510; http://www.smglink.com/bios/drycal_defender/drycaldefender.html was used. The exhaust flow from the head-out compartment was split via a manifold allowing for a simultaneous detection of NO and CO₂ (for details see Fig. 1). All gas measuring devices were regularly checked and re-calibrated using span gases of certified composition in synthetic air. Reference gases were diluted to attain concentrations in that range measured in exhaled breath. Sampling locations for gas analyses are shown in Fig. 1.

**Exhaled CO₂:** Real-time CO₂ measurements utilized non-dispersive infrared spectroscopy (Leybold-Heraeus-UM-EF-621 or X-Stream Enhanced XECK-Compact Gas Analyzer from Emerson Process Management, Hasselroth, Germany). Under the conditions chosen (air-flow rate: 0.75 L/min), the exhaled concentration of CO₂ in control rats was in the range of approximately 0.8% (8000 ppm). Control rats did not experience any time- or concentration-dependent differences in respiratory rate or exhaled NO at this level of CO₂.

**Measurements of Breathing Frequency:** Pressure fluctuations were measured in the volume displacement body compartment of the plethysmograph equipped with a wire-mesh style pneumotachograph and differential pressure transducers (MP 45 ± 2 cm m⁻¹ H₂O, Validyne) fitted shortly onto the plethysmograph. The head and body compartments were separated using a double-layer latex neck seal. Precautions were taken to avoid artifacts due to restraint and tight fitting seals around the neck. During all measurements rats were spontaneously breathing and conscious. Similar to the NO measurements pressure signals were averaged for 10 s.
The PO-NE-MAH/PLUGSYS Data Acquisition, Analysis & Archive System was used for data collection and analysis.

- Adjustment of eNO: As illustrated systematically in Figs. 2 and 3, breathing frequency and exhaled CO2 are somewhat interrelated, i.e., a higher breathing frequency results in lower exhaled concentrations of endogenously generated CO2. In conscious, spontaneously breathing rats the respiratory minute volume is limiting the amount of NO exhaled. Depending on the severity of lung injury, hypoxic pulmonary vasoconstriction and ventilation–perfusion mismatch can also occur. This bioassay-specific shortcoming can be overcome, at least in part, by the applied adjustments of exhaled NO. Perfusion-related changes were addressed by measurements of exhaled CO2, whereas the breathing frequency served as surrogate for the measurement of respiratory minute volumes. From that one may deduce an intricate relationship of CO2 and breathing frequency to the concentration of exhaled NO. This circumstance was accounted for by using the following adjustment to neutralize changes in eNO during spontaneous tidal breathing of treatment groups with affected lung mechanics and healthy controls:

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\text{NO}_{\text{adj}}(\text{ppb}) = \text{NO}_{\text{obs}}(\text{ppb}) \times \frac{\text{CO}_2^{\text{obs}}}{\text{CO}_2^{\text{min}}} \times \frac{\text{breathing rate}_{\text{min}}}{\text{breathing rate}_{\text{obs}}}
\]

whereas 'observed' represents the respective experimental group relative to the concurrent control group. A proof-of-principle study was used to verify/refute as to whether this correction can reliably be used to enhance the detectability of this biomarker.

- Proof-of-principle study: eNO was shown to be increased by nose-only exposure of rats to phosgene (30 mg/m³ × 30 min). Thereafter, these rats were exposed twice for aerosolized aminoguanidine in concentrations of 300 and 1000 mg/m³, starting at 0.5 and 4 h after exposure to phosgene. eNO and the additional endpoints for adjustments were measured 1 day post-phosgene exposure. Similar measurements were carried out in the phosgene only group (no aminoguanidine exposure). The above adjustment was considered to be physiologically validated when the eNO was decreased by an increased inhaled dose of the iNOS-inhibitor aminoguanidine (Hesslinger et al., 2009). A straightforward dose proportionality is not always expected to occur when using non-selective NOS inhibitors as the concomitant inhibition of constitutive and endothelial isoforms may increase pulmonary pressure and vasoconstriction of pulmonary vessels. This iNOS-inhibitor has been shown to be efficacious in irritant and LPS ALI models (Malaviya et al., 2012; Numata et al., 1998).

2.5. Data analysis

Reported eNO values were expressed as means ± SD, and these data were statistically evaluated using the ANOVA procedure followed by a multiple comparison Tukey–Kramer post hoc test. Statistical analyses utilized Sigma Plot 12 (Software, Systat, Point Richmond, CA). For all tests the criterion for statistical significance was set at \( P \leq 0.05 \). Apart from the aminoguanidine study with 5 rats/group, throughout all other comparisons 3 rats/group were used. This may limit the statistical power of analysis; however, does not depreciate the overall assessment of study due to the minimal SD observed.

3. Results

3.1. Optimization of bias-flow rates and temporal stability of eNO

Commonly, the default air-flow rate of 0.75 L/(kg-bw min) provides an air exchange rate 3-times the minute volume of young adult rats (Pauluhn and Thiel, 2007). Attempts were made to increase the sensitivity of eNO measurements by a lower flow-rate of synthetic air; however, at the expense of higher levels of exhaled
CO₂ within the head-out compartment (Fig. 1). This analysis compared the relationship of concentrations of eNO and CO₂ in exhaled air as well as whether temporal changes in ventilation occur as a result of the re-breathing of CO₂ at high concentrations utilizing rats dosed either with saline or LPS 1 day post-intratracheal instillation which mirrors the climax of LPS-ALI (for details see Liu et al., 2013). The air-flow rates examined are given in Fig. 2. The concentrations of CO₂ were 11,924 and 5905 ppm downstream the head-out compartment at 0.5 L/min and 1.0 L/min, respectively. In control rats, baseline data of eNO increased minimally with increasing CO₂ and lower air-flow rates whereas in LPS treated rats the expected increase was noticeable (Figs. 2 and 3). In both the control and the LPS groups, the doubling of airflow rates decreased proportionally the exhaled CO₂ and NO when corrected for the 15 ppm background level of NO in synthetic air. Relative to the control, LPS-dosed rats elaborated an increased breathing frequency which is attributed to the LPS-induced ALI rather than any CO₂-dependent stimulus (Fig. 3). The latter is expected to have caused a more time-dependent increased in ventilation. Thus, any dependence of the breathing frequency on the concentration of CO₂ was not apparent. The overall outcome is typical for a semi-fossorial species, such as rat that lives partially in burrows, which is adapted to high CO₂ environments. In concert with published evidence, at short exposure durations, rats show relatively small ventilatory response to concentrations below 50,000 ppm inspired CO₂ (Boggs, 1992).

3.2. Method comparison

Non-adjusted eNO was highest using the bag method (method-2); however, with the trade-off of additional manipulations and

Fig. 4. Exhaled nitric oxide (eNO) 1, 6, and 24 h post-LPS or saline intratracheal instillation using three different modes of exhaled air collection. Method-1: restraint rats in head-out volume displacement plethysmographs (Fig. 1), method-2: anesthesia and gas collection bags, method-3: rats without restraint in whole-body barometric plethysmographs (means ± SD, n = 3). Asterisks denote significant differences of dosed groups to the time-matched control (\(^*P<0.05\), \(**P<0.01\)).

Fig. 5. Exhaled CO₂ 1, 6, and 24 h post-LPS or saline intratracheal instillation using two different modes of exhaled air collection. Method-1: restraint rats in head-out volume displacement plethysmographs (Fig. 1), method-3: rats without restraint in whole-body barometric plethysmographs (means ± SD, n = 3). Asterisks denote significant differences of dosed groups to the time-matched control (\(^*P<0.05\), \(**P<0.01\)).
anaesthesia. Oxygen supplementation was omitted as this may have promoted oxidation of exhaled NO. The susceptibility to anaesthesia increased from one measurement to the next. Two out of three LPS-ALI rats succumbed overnight whilst control rats showed stable response patterns after each measurement (Fig. 4). This outcome demonstrates that method-2 cannot reliably be controlled in rats experiencing ALI and was abandoned accordingly. The comparison of method-1 (restraint) and method-3 (whole body plethysmography with no restraint) revealed that the best differentiation of exhaled NO between controls and LPS-ALI rats can be achieved when using method-1. Somewhat similar concentrations of exhaled CO2 (Fig. 5) were detected by either method. Changes in breathing frequencies (Fig. 6) were best reflected by volume-displacement plethysmography (method-1) accompanied by increased eNO. Differences between controls and LPS-ALI rats were identified most favorably by adjusted eNO (Fig. 4 vs. Fig. 7).

3.3. Proof-of-concept

The iNOS-inhibitor aminoguanidine was administered by inhalation post-phosgene exposure to study its impact on the concentration of exhaled CO2 (perfusion-dependent variable) and breathing frequency (ventilation-dependent variable) as well as amount of eNO. The outcome delineated in Fig. 8 demonstrates a NOS-inhibitor-dependent decrease in the adjusted eNO, whilst the concentrations of non-adjusted NO were indistinguishable amongst groups. This verifies the assumptions made in the method section, namely that the adjustment to disease-specific changes in ventilation (breathing frequency) and lung perfusion (exhaled CO2) improves the diagnostic sensitivity of this assay.

3.4. Comparison of different types of ALI

Each type of ALI seems to have its specific pattern of response. Its difference to the concurrent control is characterized best by the adjusted eNO (Fig. 9) rather than the actually measured NO (Fig. 10). The injury-related changes in exhaled CO2 and breathing frequency are shown in Figs. 11 and 12, respectively. Rats exposed to the airway irritant chlorine elaborated maximal levels of adjusted eNO relative to the non-irritant LPS and pulmonary-irritant phosgene.

Brown Norway rats that were sensitized topically and 4-times by inhalation over a time period of 2-months, a bioassay producing some hallmarks of asthma (Pauluhn and Mohr, 2005), were examined for eNO within 2–3 h after the last challenge. Naive control
and topically sensitized rats received 3 prior 30-min challenges at 80 mg TDI/m³ every 2-weeks for asthma aggravation followed by a stepped fourth challenge at 15 and 40 mg/m³. Sham exposed rats did not receive any prior challenge with TDI. At the terminal challenge, the non-sensitized but repeatedly challenged rats of the concurrent control were not statistically different from the non-challenged naïve reference group (sham) while in the sensitized groups eNO levels were significantly increased.

At the time point of data collection, the LPS- and TDI-dosed rats were in a normothermic range of body temperature whereas the rats exposed to chlorine and phosgene gas were hypothermic (30–35 °C on the exposure day).

4. Discussion

eNO is produced at elevated levels when inflammation within the respiratory tract is present. Therefore, it is considered to be a suitable biomarker to judge the degree of acute lung injury following exposure to irritant toxicants. Likewise, eNO may also serve as early biomarker to adjust treatment strategies. Therefore, the focus of this methodological comparison was to find a method suitable to identify different types of ALI prior to their clinical manifestation in order to categorize disease severity. Similarly, the measurement of eNO can guide the applied effectiveness of early treatment and countermeasure thus offering a possibility for guiding the selection of therapy, improving dosage and dosing frequencies.

In rat models, the simplicity of the measurement of eNO is both its strength and weakness. Diffusion and partition of NO take place at proximal and distal locations of the lung. Increasing losses of locally produced NO may occur due to axial diffusion in the alveolar region because of its large surface area and limited diffusion air–blood barrier. The large pool of blood (hemoglobin) available in this region provides an almost infinite sink to scavenge NO. The number of variables involved in the exhalation of NO in humans has been dealt with in details elsewhere (Condorelli et al., 2007). These variables even increase in complexity when using small laboratory rodents. Exhalation maneuvers over the entire range of vital capacity can readily be managed in humans but requires anesthesia, extensive instrumentation and equipment in small laboratory rodents. Thus, any comparable simplicity of measurement of eNO can only be achieved when using non-cannulated, conscious tidal breathing rats. Moreover, with increased lung injury changes in lung mechanics and diffusing capacity occur with modified ventilation and efflux of eNO. Therefore, the diagnostic sensitivity of adjusted eNO is superior to non-adjusted NO.

Inhalation exposure of rats to irritant substances may cause hypothermia (Gordon et al., 2008). The small tidal volume of rats (1.5 mL/breath, for breathing frequencies see Fig. 3) requires sufficiently high carrier air-flow rates to allow the simultaneous measurement of the exhaled NO and CO₂ utilizing minimized dead-space volumes. As shown in Figs. 9 and 10, when adjusting eNO for "alveolar CO₂" and "exhalation rate", most of these rat-specific shortcomings of analysis can be overcome by using method-1. Hypothermia correlates with depressed metabolism (Gordon et al., 1988; Gordon, 1990,1993), changes in the alveolar ventilation-perfusion relationship and lung mechanics (Lai et al., 1978; Das and Blanc, 1993). It cannot be ruled out that hypothermia
can also affect the production and efflux of NO. Therefore, the measurement of "alveolar CO₂" may also be suitable to adjust for body temperature-related differences between rats inflicted with ALI and healthy controls.

Despite the higher sensitivity of method-2 (orotracheal cannula, superficial anesthesia, and exhaled breath collection in a gas bag), the objectives of study were met best by method-2. The adjustment of exhaled NO to exhaled CO₂ and breathing frequency to the concurrent control enhanced the diagnostic sensitivity of both method-1 and method-3. Accordingly, method-1 was considered superior to method-3 as its two-compartment configuration shortened response times and minimized potential interference of measurements by off-gassing substances from urine or feces. Therefore, the comparison of different types of lung injury utilized solely this method.

The comparative analysis of parenchymal inflammation (LPS) with irritant ALIs typical of airway injury (chlorine) and acute lung edema (phosgene) revealed distinct response patterns in rats early after exposure. For the edemogenesis-inducing phosgene, adjusted eNO is amongst the earliest predictive non-invasive biomarkers (Fig. 9) relative to the invasive endpoints BAL-protein, increased lung weights, and hemocoagulation as detailed elsewhere (Li et al., 2013). Significantly elevated adjusted eNO relative to the equally TDI-challenged naïve control rats was also observed in the Brown Norway rat asthma model (Fig. 9). Alike humans, disocyanates elicit preferentially a delayed allergic response in this animal model. Transient delayed responses commonly occur within the range 1.5–3.5 h post-inhalation provocation (Pau1uhn, 2008). The absence of any consistent TDI-provocation dose-dependence of the adjusted eNO is likely to be caused by any mismatch of the climax of the transient delayed onset change in lung function and measurement of adjusted eNO.

Notably, all lung inflammation-inducing substances with differing etiopathologies and major sites of injuries resulted in elevated levels of adjusted eNO. Measurements in phosgene-exposed rats were complemented by treatment with the iNOS-inhibitor aminoguanidine which caused a dose-dependently reduced adjusted eNO (Fig. 8). This corroborates the need for a physiology-based adjustment of actually measured eNO to account for disease-specific changes in perfusion and ventilation, i.e., variables that affect the localized, intraluminal concentration of NO as well as its flux of exhalation.

Collectively, this study compliments the prevailing clinical evidence that eNO is an invaluable, non-invasive biomarker of early acute lung injury suitable to identify the degree and possibly also the site of major injury. Therefore, it fulfills the objective of study to direct and stratify any ensuing treatment regimen. The diagnostic sensitivity of this biomarker is markedly improved by physiology-based adjusted eNO, allowing a more systematic research on different types of ALI (e.g., septic inflammation, airway and alveolar irritation-related inflammation) with less experimental animals as required by conventional terminal methodologies. Accounting for the at least 3-fold dilution of exhaled air used in this rat bioassay, to make dynamic measurements during tidal breathing possible, one may conclude that similar types of measurements might be at least 3-times more sensitive in humans.

**Fig. 9.** Analysis of adjusted eNO 5–6h and 24 h post single LPS intratracheal instillation, chlorine gas, and phosgene gas of Wistar rats nose-only inhalation exposure. Air nose-only exposed Wistar rats served as control (n = 3). TDI vapor was studied in skin-sensitized and inhalation re-challenged Brown Norway rats (n = 4). Measurements of eNO were made 2–3 h post-inhalation challenge with TDI. Bars represent the means ± SD of non-sensitized by re-challenged control Brown Norway rats. Details of this Brown Norway rat asthma model were published elsewhere (Pau1uhn, 2008). Asterisks denote significant difference to the concurrent control (*P < 0.05, **P < 0.01).
Fig. 10. Analysis of eNO 5–6 h and 24 h post single LPS intratracheal instillation, chlorine gas, and phosgene of Wistar rats nose-only inhalation exposure. For further details see legend of Fig. 9.

Fig. 11. Analysis of exhaled CO₂ 5–6 h and 24 h post single LPS intratracheal instillation, chlorine gas, and phosgene of Wistar rats nose-only inhalation exposure. For further details see legend of Fig. 9.
Conflict of interest

This study has been performed in context with the thesis of FL. There is no conflict of interests. This thesis is supported by a Ph.D. program grant from Bayer HealthCare (BHC), Wuppertal, Germany, and Beijing universities domestic and abroad cooperation training base program, Beijing, China. There is nothing to disclose by the remaining authors.

References


Fig. 12. Analysis of breathing frequency 5–6 h and 24 h post single LPS intratracheal instillation, chlorine gas, and phosgene of Wistar rats nose-only inhalation exposure. For further details see legend of Fig. 9.


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