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

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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 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 $CO_2$ 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.



## 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

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vasoconstriction (Beckman and Kppenol, 1996; Hesslinger et al., 2009). The expression of these isoforms 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; Brindicci 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- and 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 toluene 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; toluene 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/Crl 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.sourceoecd.org). 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 (toluene 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 $CO_2$ 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 septic ALI. eNO was measured on days 0 (1 and 6 h post-IT) and 1 (approximately 24 h 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).