Exhaled nitric oxide measurement to monitor pulmonary hypertension in a pneumonectomy-monocrotaline rat model

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PULMONARY ARTERIAL HYPERTENSION (PAH) is a progressive disease, leading to early death if left untreated (9, 19). It is marked by increased vascular resistance in the distal pulmonary arteries as a consequence of vasoconstriction, proliferative remodeling of the pulmonary vasculature, inflammation, and thrombosis. Impaired production of vasodilators such as prostacyclin and nitric oxide (NO) has been proposed to be involved in disease pathogenesis (12). NO plays a major role in vascular homeostasis, smooth muscle cell proliferation and migration, platelet aggregation, and leukocyte adhesion on the endothelium (36). Low expiratory NO concentrations have been found in PAH patients (10, 16, 35) and even an inverse relation between exhaled NO, its biochemical reaction products, and mean pulmonary arterial pressure (mPAP) has been shown (16). Although there are established and uniform measurement techniques for the assessment of exhaled NO in humans (1), noninvasive measurement in animals is challenging, since the expected NO concentration lies close to the quantification threshold. Most groups solved this problem by adding an accumulation process (2, 6, 21, 30, 32), varying in time and setup. At such low concentrations the error by sampling NO for its quantification alters the concentration in the measuring chamber.

The aim of our study was to establish a reliable and reproducible method for fractional exhaled NO (FeNO) measurement in a rat model of PAH, to compare the obtained exhaled NO values to an already-published method, and to test whether the novel technique is able to trace even minimal effects of an NO-regulating therapy with the NO-substrate L-arginine and the cofactor tetrahydrobiopterin (BH4).

MATERIALS AND METHODS

Animal model. This study was approved by the Austrian Ministry for Science and Research and was performed according to the Helsinki convention for the use and care of animals. A total of 62 male Sprague-Dawley rats [weight: 412.15 ± 20.74 g; age: 29 ± 20.74 days (29)] underwent unilateral pneumonectomy and simultaneous implantation of a DSI PA-C40 pressure catheter in the common pulmonary artery trunk (22, 33). For anesthesia the animals received 2% xylazine (4 mg/kg)/ketamine (100 mg/kg) and were intubated. After connection to a small animal ventilator (Biegler, Mauerbach, Austria) the animals received 1.5% isoflurane at an inspired oxygen fraction of 0.8 and a respiratory volume of 1.5 l/min. All animals were given a single shot of enrofloxacin (Baytril, Bayer, Vienna, Austria) as perioperative antibiotic prophylaxis. For placement of the DSI catheter a refined technique was used. In brief, the catheter tip was directly inserted into the pulmonary artery after resection of the left lung via a lateral thoracotomy (27). The excised lung was utilized as nondiseased control tissue for all experiments. Hemodynamic data were gained and processed by DSI 4.2 ART software system (DataScience International, Boston, MA). In every rat, mPAP and heart rate were recorded over 5 min per hour, and a mean value for each day was calculated. PAH was defined as mPAP > 25 mmHg. Rats were provided tap water and common rat chow (ssniff, Soest, Germany) ad libitum. Seven days after the surgical intervention, PAH was induced by subcutaneous administration of 60 mg/kg monocrotaline (MCT; Sigma, Vienna, Austria). From day 35 onward, a daily combination of L-arginine (300 mg/kg) and FeNO was assessed. After 35 days, animals were randomized to receive either oral L-arginine (300 mg/kg) (28) (Sigma) and BH4 (20 mg/kg) (kindly provided by AOP Orphan Pharmaceuticals, Vienna, Austria) ad libitum. Seven days after monocrotaline exposure (11) daily over a period of 14 days.

Using the modified technique, we found an inverse correlation between exhaled NO and pulmonary pressures before (r = −0.366, P = 0.043) and after MCT (r = −0.363, P = 0.038) as well as after therapy administration (r = −0.657, P = 0.02). Our modified technique proved robust in a rodent model, since valid and reproducible data were gained and showed an inverse correlation between exhaled NO and mPAP, whereas the existing method did not.

fractional exhaled nitric oxide; noninvasive measurement; rat; monocrotaline

References

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orogastric gavage. To prevent aspiration, animals remained in an upright position until they regained consciousness. FeNO values were determined before the administration of MCT, on day 35 (at which point all included animals had developed PAH), and at the end of observation.

**Nitrile oxide measurement.** A chemiluminescence analyzer (CLD 66, Eco Physics, Duernten, Switzerland) with a sensitivity of 0.5 ppb and a withdrawal rate of 100 ml/min was used to measure gas phase NO. For the measurement of FeNO, single spontaneously breathing rats were placed in a whole body plethysmography (WBP) chamber (3.9 liters, PLY 3213 Buxco Research Systems, Wilmington, DE). The WBP chamber was connected to the Denox88 system (Eco Medics, Duernten, Switzerland), which produces and carries a continuous flow of NO-free air (300 ml/s) into the WBP chamber and into the chemiluminescence analyzer. Measurements were recorded by excel Macro (cldx2xls, Jörg Hoffmann, ECO PHYSICS). Past elimination of NO from the WBP chamber was achieved by an outlet located at the top of the chamber. Sampling was planned after the setup described by Ahmad et al. (2). The WBP chamber was flushed with NO-free air and was sealed when NO levels were back to baseline. The exhaled breath of the rat was collected for 300 s. This would be the approximate time that the entire gas volume inside the WBP chamber would have been inhaled and exhaled by the rats. Then the outflow stopcock was opened for NO measurement, and the recording continued for another 300 s.

**Modified concept of nitric oxide measurement.** In undisturbed closed systems (like the WBP chamber) concentrations display an exponential curve at steady accumulation. However, because of the instability of NO and time limitations, this approximation value cannot be used in practice. In the period during which NO is allowed to accumulate in the plethysmograph, the measured NO concentration vs. time produces a near-linear relationship. In addition, after opening the closed system, the true NO concentration is underestimated because of its constant removal for sampling. To overcome this technical issue, we first determined the NO values (parts per billion per hour, ppb/h) as described above. The first 30 s of transient condition at the beginning of recording were excluded to reduce the bias caused by the inertia of the measuring system. By averaging the NO levels of the remaining 270 s, two points were generated through which a partial regression line was laid to determine the standard slope (FeNOstand) (Fig. 1, slope 3). For the mathematical modification FeNOstand was used for extrapolation and determination of the calculated initial chamber concentration (cENOi; Fig. 1, point 4). The oscillation of the baseline values was evened out by average determination and a zero point was created. The modified slope (FeNOmodif; Fig. 1, slope 5) was calculated by laying a partial regression line through the zero point and cENOi, receiving a steeper slope than FeNOstand.

We presume that this increase represents the successful elimination of bias created by dilution and oscillating values that may be crucial at such a low concentration.

To validate our modified method we applied it to a cohort of six untreated rats (no pneumonectomy, no MCT) in an intervention that predictably affects NO production. L-N\textsuperscript{G}-nitroarginine methyl ester (L-NAME hydrochloride, Bertin Pharma, Montigny-le-Bretonneux, France) nonselectively inhibits NO synthase and therefore reduces NO exhalation in rodent models (4, 17). L-NAME was administered by two intraperitoneal injections (2 × 100 mg/kg) with an interval of 30 min (17, 32). Prior to and 30 min after L-NAME application, FeNO was assessed by both the standard and the modified method.

**Immunohistochemistry and morphometry.** At the end of the observation period animals were anesthetized and euthanized by intracardial thiopental bolus injection. The remaining lung was removed for further analysis. Endothelial NO synthase (eNOS) mRNA expression was measured from nitrogen-snap-frozen pulmonary tissue samples using the RNeasy fibrous tissue kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany) and determined by real-time PCR using eNOS primer Rn02132634_S1 (7). eNOS expression was visualized in immunohistochemically stained lung tissue slides (anti-eNOS antibody ab66127, Abcam, Cambridge, UK) and intradividually compared with the nondiseased sample. We chose third order pulmonary arteries with an outer diameter <350 μm (5), since they were relatively easy to detect and displayed all features of hypertensive pulmonary arteries and compared them to vessels ≥350 μm. Percent medial wall thickness (%MWT) was calculated as described elsewhere (25). In brief, %MWT was calculated by the following equation: %MWT = [(medial thickness × 2)/external diameter] × 100. Six vessels of each section were randomly chosen and analyzed by two independent observers using ImageJ 1.46 (National Institutes of Health, Bethesda, MD).

**Statistical analysis.** Results were compared using SPSS 19 Statistic software (IBM, Armonk, NY). An unpaired Student’s t-test was used for comparison of data between methods. Data generated by both methods were assessed by Spearman correlation analysis and linear regression analysis. A P value of <0.05 was regarded as significant.

**RESULTS**

**Animal model.** Twenty-seven rats had to be excluded from our follow-up (perioperative death in 9 animals, death due to wound infection in 2 animals, death attributed to PAH in 9 rats, temporary malfunction of the NO measuring apparatus in 7 rats). Of the remaining 35 animals, two did not develop PAH and were also excluded from this analysis. The mPAP at
baseline (postpneumonectomy) was 17.19 ± 9.62 mmHg, which increased to 53.13 ± 10.63 mmHg after 28 days of MCT exposure (P < 0.001; Table 1).

**Modified FeNO measurement.** Basal standard NO levels were 16.16 ± 7.19 ppb/h whereas basal modified values were 21.21 ± 8.27 ppb/h. Twenty-eight days after MCT application, modified NO levels increased by 3.17 ± 6.17 ppb/h after establishment of PAH (P = 0.01; Fig. 2) whereas standard NO levels remained unaffected (−1.62 ± 8.35 ppb/h; P = 0.2). Relying on the conventional FeNO assessment (2), neither correlation could be identified between FeNO and pulmonary pressures before (r = −0.282, P = 0.124) nor after (r = −0.258, P = 0.148) development of PAH. After modification of FeNO assessment, we found a moderate inverse correlation between FeNO and pulmonary pressures before (r = −0.366, P = 0.043) and after (r = −0.365, P = 0.038) development of PAH (Fig. 3).

In a linear regression model, the conventional technical setup failed to predict pulmonary pressure before ($R^2 = 0.076$, $P = 0.133$) and after ($R^2 = 0.059$, $P = 0.175$) the application of unilateral pneumonectomy and MCT injection (P/MCT). With the modified setup, there was a moderate relation between FeNO and mPAP before ($R^2 = 0.118$, $P = 0.059$) and a significant association between FeNO and mPAP ($R^2 = 0.213$, $P = 0.007$) after P/MCT.

**Table 1. Animal characteristics**

<table>
<thead>
<tr>
<th></th>
<th>pre-P/MCT (day 7)</th>
<th>post-P/MCT (day 35)</th>
<th>post-VH (day 49)</th>
<th>post-TH (day 49)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>33</td>
<td>33</td>
<td>11</td>
<td>12</td>
</tr>
<tr>
<td>TBW, g</td>
<td>412.15 ± 46.30</td>
<td>414.61 ± 59.79</td>
<td>422.97 ± 69.13</td>
<td>402.00 ± 77.52</td>
</tr>
<tr>
<td>HR, beats/min</td>
<td>343.5 ± 34.30</td>
<td>349.02 ± 34.40</td>
<td>432.09 ± 60.59</td>
<td>399.57 ± 71.60</td>
</tr>
<tr>
<td>BR, breaths/min</td>
<td>104.81 ± 21.82</td>
<td>120.73 ± 29.77</td>
<td>306.57 ± 16.57</td>
<td>332.97 ± 16.50</td>
</tr>
<tr>
<td>mPAP, mmHg</td>
<td>17.19 ± 9.62</td>
<td>53.13 ± 10.63</td>
<td>51.1 ± 17.6</td>
<td>47.2 ± 16.2</td>
</tr>
<tr>
<td>FeNOstand, ppb/h</td>
<td>16.16 ± 7.19</td>
<td>16.33 ± 6.50</td>
<td>18.81 ± 4.11</td>
<td>18.31 ± 11.18</td>
</tr>
<tr>
<td>eNOS, logRQ</td>
<td>0.00 ± 0.57</td>
<td>n. a.</td>
<td>0.60 ± 0.86</td>
<td>−0.84 ± 0.84</td>
</tr>
</tbody>
</table>

pre-P/MCT, prior to monocrotaline application; post-P/MCT, after pneumonectomy and monocrotaline application; post-VH, after therapy administration; post-TH, after vehicle administration; N, number of animals; TBW, total body weight; HR, heart rate; BR, breathing rate; mPAP, mean pulmonary arterial pressure; FeNOstand, slope of exhaled nitric oxide values generated with the standard method; FeNOmodif, slope of exhaled nitric oxide values generated with the mathematical modification; eNOS, endothelial nitric oxide synthase results from real-time PCR. n. a., not available. For $P$ values, see text.
Therapy administration led to a significant decrease of mPAP (−7.8 ± 15.4 mmHg, \( P = 0.003 \)) and a trend toward increasing FeNO measured by the modified setup (\( P = 0.068 \)). Furthermore, a significant inverse correlation was shown between mPAP and FeNO (\( r = −0.657, \ P = 0.02 \)).

FeNO measurement after \( \text{L-NAME} \) application. Intraperitoneal application of \( \text{L-NAME} \) led to a significant decrease of NO after 30 min. The mean baseline value as measured by the standard method was 9.87 ± 1.87 ppb/h and decreased to 7.17 ± 2.29 ppb/h (\( P = 0.05 \)). In the modified method baseline NO was 15.82 ± 1.29 ppb/h and decreased to 11.3 ± 2.15 ppb/h (\( P = 0.001 \)).

eNOS expression. As supported by prior findings (20, 31) our data showed eNOS mRNA upregulation in diseased vs. healthy contralateral lungs [0.603 \( \log^{10} \) relative quantity (RQ) after P/MCT, \( P = 0.03 \)]. Therapy administration led to a significant eNOS downregulation to −0.839\( \log^{10} \) RQ (\( P = 0.008 \)). Staining showed impaired eNOS expression in the endothelium of P/MCT exposed pulmonary vasculature (Fig. 4). The ratio of eNOS-positive endothelial cells to the total number of cells per vessel was significantly lower in PAH (0.99 ± 2.92%) compared with native lungs (8.32 ± 10.49%, \( P < 0.001 \)) whereas %MWT was significantly higher (PAH: 23.45 ± 2.6% vs. native: 14.33 ± 1.65%, \( P < 0.001 \)). These changes were even more prominent in smaller pulmonary arteries (outer diameter <350 \( \mu \)m, mean 166.13 ± 143.67 \( \mu \)m) characterized by a higher %MWT (26.65 ± 4.56%) than larger vessels (20.11 ± 3.72%, \( P < 0.001 \)) and correlating with less eNOS-positive cells (\( r = −0.491, \ P < 0.001 \)). Therapy administration did not significantly improve %MWT or eNOS ratio.

DISCUSSION

By mathematical modification of a standard measuring technique we developed a novel method of NO measurement in the P/MCT rat model. Because of a better correlation with hemodynamic parameters our concept seems to be promising in terms of a superior noninvasive preclinical evaluation of pathophysiology and therapy in PAH.

Since 2005 the measurement of FeNO in human subjects is defined by the ERS/ATS workshop (1). In animals and especially small rodents these guidelines cannot be adapted, because of the high mortality of invasive ventilation in pulmonary hypertensive rats.

We chose an optimal setup of NO-free air for flushing the animal chamber, followed by a short accumulation time paired with a withdrawal of a small volume of gas and a long acquisition phase. We excluded the first 30 s of recordings to eliminate the bias of dead volume and evened out oscillations in measurement to receive a linear calculated slope predicting the accumulation of NO. This calculation allows a standardization of the different accumulation times and setups used in other publications [e.g., a NO concentration of ~5 ppb after 20 min of accumulation (6), compared with 5.38 ppb when using our standard measurement in the same setup]. After applying our modification a value of 7.07 ppb would be read out. This result suggests that measurement inaccuracy and error can be compensated by mathematical modification only.

A decreased NO production has been proposed to be an important prognostic factor in the course of PAH (16, 23). In accordance, our study also found an inverse correlation between FeNO and mPAP with the new modified technique. However, the overall NO increased after P/MCT. This might represent an unspecific surrogate marker for the inflammatory response to MCT application as seen in asthma (10, 34). In our rat study low NO values are associated with high pulmonary pressures, so these findings might predict changes in pulmonary hemodynamics also in the P/MCT model. The observed differences in correlation coefficients (\( r \)) are explained by a distortion caused by the experimental setting. At comparable correlations, differences in scattering of measured variables may be responsible for this phenomenon. At baseline (before
any manipulation), pulmonary pressures in study animals are homogenous with low scattering. After therapy, individual mPAPs display a more heterogeneous distribution. As a reflection of these differences in scattering, correlation coefficients increased from −0.366 at baseline to −0.657 after therapy.

As previously described MCT has deleterious effects on pulmonary vasculature (24), which is reflected by a low number of eNOS-positive cells (11) and by the increasing media thickness of affected arteries (26). In our model, %MWT increased after P/MCT application, proving that PAH could be effectively established. After therapy with the NO substrate L-arginine and the cofactor BH4, NO levels rose and consequently eNOS mRNA was downregulated. No reduction of %MWT was observed, suggesting that the therapeutic effect is predominantly because of NO-induced vasodilation and to a lesser extent to vascular remodeling.

In humans, orally administered L-arginine led to a significant increase of NO concentration in exhaled breath (13). A single dose of BH4 significantly increased FeNO in hypoxic and normoxic rats (14). In line with these observations, a combination of both led to a slightly higher NO concentration in our animal model.

Study limitations. Animal studies might not fully represent the pathology in humans. However, our data correspond to results found in PAH patients, so that the conclusions seem to be transferable to human disease.

Our modification itself has not been independently verified. Nevertheless our simple and reproducible FeNO measurement data correlated better with mPAP values than the standard measurements. Moreover, we were able to prove this methodological superiority by evaluation in a model of eNOS inhibition by L-NAME. Introduction of a new diagnostic technique requires both positive and negative predictive values. Defining a cutoff value for determination of true positive and negative calls would result in loss of information compared with simple correlations of continuous variables. Therefore a higher case number would be required for valid results. In more extended experiments we subjected 11 additional animals to combined therapy (data not shown). We defined therapeutic success as reduction of mPAP below a cutoff value of 30 mmHg. This setting, we calculated a positive predictive value of 88.9% [95% confidence interval (CI) 67.2–96.9%] and a negative predictive value of 18.8% (95% CI 6.6–43.0%). This yield a large CI, which can only be narrowed by increasing the number of animals. We calculated that for a clinically acceptable CI of ~10% an ethically unjustifiably high total number of 200 animals would be necessary.

Another shortcoming is the lack of a treatment group, which demonstrably improved PAH. A number of therapeutic strategies have been used that yield better resolution of MCT-induced PAH (3). By reviewing the literature on the efficacy of drugs licensed for the treatment of human PAH we found that the treatment effects of most substances in the rat MCT model were comparable to the mean reduction of mPAP in our study [e.g., mean reduction of mPAP ~10 mmHg with either bosentan or sildenafil vs. 6 mmHg in our setting (8)]. Even if a few more rats had reached the cutoff of 30 mmHg, the total number of animals would only be marginally reduced. In addition, our model of PAH rather reflects advanced disease since it combines MCT application with unilateral pneumonectomy. It is unknown whether the substances referenced above (8) would achieve the same effect in this more severe stage of disease.

Conclusion. We successfully established an NO measurement method that led to valid and reproducible data in a rat model of PAH. This new technique is based on mathematical modification of an established NO measuring procedure and leads to more reliable data by diminishing measuring errors. An inverse correlation between FeNO and mPAP before and after P/MCT application and after a combined L-arginine and BH4 administration was shown. This relation can be used to noninvasively investigate the course of P/MCT-induced PAH and response to therapy in rats.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


