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Decreased Whole Body Endogenous Nitric Oxide Production in Patients with Primary Pulmonary Hypertension

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Key Words

Mass spectrometry · Nitric oxide · Pulmonary hypertension

Abstract

Impaired pulmonary release of nitric oxide (NO) is one of the characteristic phenotypic changes of vascular cells in pulmonary hypertension. The aim of this study was to determine nitric oxide synthase (NOS)-dependent whole body NO production in patients with primary pulmonary hypertension. NOS-dependent whole body NO production was assessed by giving an intravenous infusion of *L*-[$^{15}N]_2$-arginine (50 μmol/min for 30 min) and measuring isotopic urinary enrichment of ^{15}N-nitrite and ^{15}N-nitrate. Four female patients with no signs of infection were recruited and compared with 6 age-matched control subjects. Mean 12-hour excretion of ^{15}N-nitrite and ^{15}N-nitrate in the total urine over 36 h was smaller in patients than in control subjects (57.2 ± 27.6 vs. 229.1 ± 65.2 nmol/mmol creatinine, $p < 0.01$, Mann-Whitney U test, respectively). Neither mean 12-hour excretion of ^{14}N-nitrite and ^{14}N-nitrate (51.6 ± 10.0 vs. 72.4 ± 10.0 μmol/mmol creatinine, $p = 0.3$) nor glomerular filtration rates (84.5 ± 15.8 vs. 129.7 ± 16.0 ml/min, $p = 0.1$) were different between patients and control subjects. Our results suggest that either basal NOS-dependent whole body NO production is impaired or excess NO metabolism occurs in patients with primary pulmonary hypertension.

Introduction

The extensive phenotypic changes of pulmonary vascular cells cause the characteristic structural and physiological abnormalities in pulmonary hypertension [1]. While basal rate of endothelial nitric oxide (NO) production modulates pulmonary vascular tone [2] the role of NO in the pathogenesis of pulmonary hypertension remains controversial.

While decreased endothelial nitric oxide synthase (eNOS) expression has been reported [3], almost all studies to date have used exhaled breath NO and biochemical products of NO to infer the involvement of NOS in pulmonary hypertension [4–6]. However, basal levels vary according to diet, lifestyle and kidney function. Many of the problems associated with using point estimates of NO, nitrite and nitrate concentrations can be overcome

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by using the conversion of a subsystemic dose of non-radioactively labelled *L*-[^{15}N]$_2$-arginine to ^{15}N-nitrite and ^{15}N-nitrate in man [7].

The aim of this study was therefore to determine if NOS-dependent whole body NO production is affected in patients with primary pulmonary hypertension. We measured the formation of ^{15}N-nitrite and ^{15}N-nitrate in urine after an intravenous dose of *L*-[^{15}N]$_2$-arginine as indicator of whole body endogenous NO production in vivo and compared patients with age- and sex-matched control subjects.

Patients and Methods

Four female patients (43 ± 3 years old) referred to the Sheffield Pulmonary Hypertension Service were included in the study. None of the patients were on non-steroidal anti-inflammatory drugs in the week before the study. Six healthy, non-smoking female volunteers (36 ± 4 years old) were used as a control group. Subjects abstained from eating nitrite- and nitrate-containing foods, such as green vegetables and preserved red meat. All provided written informed consent and the study had received approval of the South Sheffield Ethics Committee.

Venous blood samples were taken for liver function tests, basal nitrite and nitrate measurements, and determination of background isotope ratios of urine nitrite and nitrate concentrations. *L*-[^{15}N]$_2$-arginine (purity > 98%, Mass Trace, USA) was dissolved in physiological saline (0.9%; Baxter Healthcare Ltd., Thetford, UK). Pharmaceutical grade *L*-[^{15}N]$_2$-arginine was infused at a subsystemic dose of 50 μmol/min for 30 min. Total dose of arginine infused was therefore chosen so that normal circulating levels would not be affected [7]. Total urine collection was collected over 48 h, from 12 h before until 36 h after infusion. Serum and urine samples were frozen and stored at –20°C until analysis.

The ratio of urinary ^{15}N-nitrate to ^{14}N-nitrate was determined in 100-μl aliquots of urine using a method adapted from Tsikas et al. [8] which involved reduction to ^{15}N-nitrite by pre-treatment with cadmium. Spiking a sample of a known amount of ^{14}N-nitrate with ^{15}N-nitrate (98 atom %, Aldrich, UK) demonstrated that our technique standard curve was linear over the range of empirical enrichments from 0 to 5% ($r^2 = 0.996$). The coefficient of variation, within assay precision, was 0.5% on replicate measures. The Griess assay was used to measure the total amount of urinary nitrate in each of the 12-hour samples. Nitrate was reduced to nitrite by pre-treatment with cadmium. This assay was linear up to 500 μmol/l with a coefficient of correlation of 0.998 and a limit of detection of 1.9 μmol/l. Both our recoveries were greater than 80%. Urinary and blood creatinine concentrations were measured by the Clinical Chemistry Department of the Royal Hallamshire Hospital.

Data are given as means ± SEM or as a box plot. Median values are presented as a line inside the box. 25th and 75th percentiles are given in each box. Top and bottom of each bar are the 90th and 10th percentiles, respectively. Non-parametric analysis was applied and differences between groups were analysed by the Mann-Whitney U test (SPSS for Windows, version 12.0, SPSS, Chicago, Ill., USA). Values of $p < 0.05$ were considered significant.

Table 1. Hemodynamic parameters and demographic data for the patients with primary pulmonary hypertension

Age years	CO l/min	mPAP mm Hg	Whole body NO production nmol ^{15}N-nitrite and nitrate/ mmol creatinine	GFR ml/min	Medications
37	6.4	30	0–12h: 83.7 12–24h: 326.4 24–36h: 81.4	78.4	diuretics diltiazem
40	2.9	45	0–12h: 130.0 12–24h: 0 24–36h: 0	58.2	diuretics amlodipine
46	4	69	0–12h: 50.4 12–24h: 0 24–36h: 0	71.2	diuretics diltiazem
48	6.4	62	0–12h: 8.8 12–24h: 5.1 24–36h: 0	130.1	diuretics nifedipine

mPAP = Mean pulmonary artery pressure; GFR = glomerular filtration rate.

Results

The patients had primary pulmonary hypertension, with a mean value for the group's mean pulmonary artery pressure of 51.5 ± 8.8 mm Hg (table 1). No patient or normal subject had systemic hypertension (systolic blood pressures < 140 mm Hg and diastolic blood pressures < 90 mm Hg). All patients were receiving oral anticoagulants to achieve an international normalised ratio >2.2 and were also taking diuretics and calcium channel blockers (diltiazem, n = 2, amlodipine, n = 1, or nifedipine, n = 1).

Mean 12-hour excretion of ^{15}N-nitrite and ^{15}N-nitrate in the total urine over 36 h was smaller in patients than in controls, i.e. 57.2 ± 27.6 vs. 229.1 ± 65.2 nmol/mmol creatinine ($p < 0.01$, Mann-Whitney U test), respectively (fig. 1). Mean 12-hour excretion of ^{14}N-nitrite and ^{14}N-nitrate was not different between patients and control subjects (51.6 ± 10.0 vs. 72.4 ± 10.0 μmol/mmol creatinine, p = 0.3). There was no correlation between hemodynamic parameters and whole body nitric oxide synthesis in this small cohort of subjects (table 1). Glomerular filtration rates were similar between patients and control subjects (84.5 ± 15.8 vs. 129.7 ± 16.0 ml/min, respectively, p = 0.1).

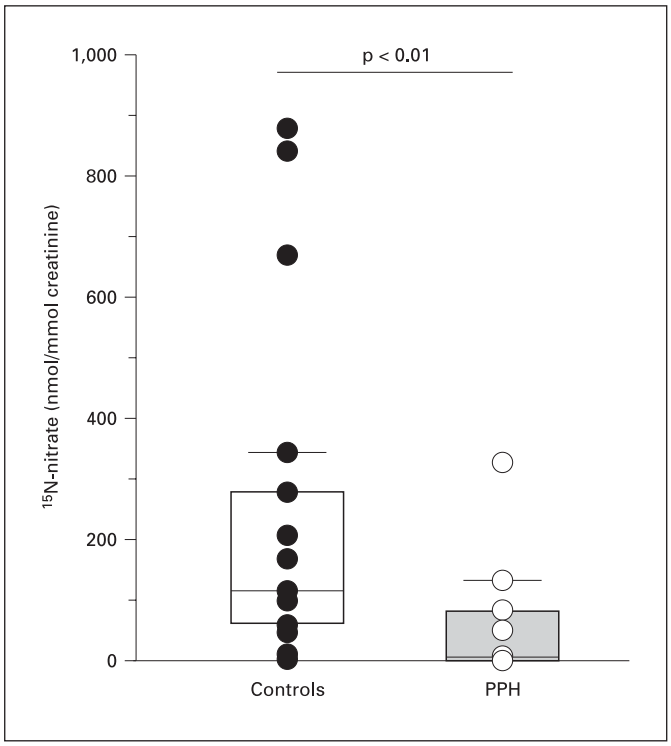

Fig. 1. Urinary excretion of ^{15}N-nitrate in female control subjects (n = 6) and patients with primary pulmonary hypertension (PPH, n = 4) after an intravenous injection of *L*-[^{15}N]$_2$-arginine (50 μmol/min for 30 min). Medians, and 75th and 25th percentiles are given in each box. Top and bottom of each bar are the 90th and 10th percentiles, respectively (Mann-Whitney U test).

Discussion

In the present study, we tested the hypothesis that NOS-dependent whole body NO production was affected in patients with primary pulmonary hypertension. We have demonstrated that it was decreased in this patient population indicating an abnormal balance between NO enzymatic synthesis and its rate of consumption. An important strength of this study is that our results are independent from exogenous sources of NO, nitrite and nitrate.

The inability of point estimates of concentration of NO in breath or its biochemical products in serum to detect changes in rates of NO production is supported by earlier work. Breath NO may be both elevated [5] or decreased [3, 4] in pulmonary hypertension. Serum NO products do not appear to be affected when subjects with pulmonary hypertension are not controlled for dietary intake of nitrite and nitrate [4, 5].

We saw no relationship between age and endogenous NO production. Neither reduced renal function nor nitrate excretion rates accounted for the differences we observed. Treatment with dihydropyridine calcium antagonists, dialtizem, amlodipine and nifedipine, which can cause NO release from endothelium [9, 10] did not elevate the rates of NO production to normal levels in our patients.

Our results can be explained by both decreased NOS activity or increased NO, nitrite and nitrate rate of consumption. In support of the former hypothesis, decreased NOS expression has been reported in patients with pulmonary hypertension [6]. Furthermore, recent advances from Pearson et al. [11] suggest that functional polymorphism in carbamoyl-phosphate synthetase, a rate-limiting enzyme in arginine synthesis, might be associated with the development of pulmonary hypertension and that the resulting low serum arginine levels may decrease NOS activity. Finally, elevated serum concentration of asymmetric dimethylarginine, an endogenous inhibitor of NOS, has been reported and may inhibit NO production in pulmonary hypertension [12].

Impairment in the NO metabolism may also be involved in the pathogenesis of pulmonary hypertension [13]. Indeed, Bowers et al. [14] have recently reported formation of nitrotyrosine in the lungs of patients with severe pulmonary hypertension. While over 70% NO is oxidised to nitrite and nitrate and excreted in the urine over 48 h [15], formation of nitrotyrosine or other nitrated proteins might shunt NO consumption away from terminal oxidation to nitrite, which acts as source of NO in the vasculature [16–18].

In conclusion, the measurement of the rate of NO production using conversion of [^{15}N]$_2$-arginine to ^{15}N-nitrate and ^{15}N-nitrite offers a specific measure of NOS-dependent whole body endogenous NO production. The need for further investigations is emphasised by our findings to explain how whole body endogenous NO production is decreased in patients with primary pulmonary hypertension.

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References

1 Humbert M, Morrell N, Archer S, Stenmark K, MacLean M, Lang I, Christman B, Weir EK, Eickelberg O, Voelkel NF, Rabinovitch M: Cellular and molecular pathobiology of pulmonary arterial hypertension. J Am Coll Cardiol 2004;43(12 suppl S):S13–S24.

2 Cremona G, Wood AM, Hall LW, Bower EA, Higenbottam T: Effect of inhibitors of nitric oxide release and action on vascular tone in isolated lungs of pig, sheep, dog and man. J Physiol 1994;481:185–195.

3 Giaid A, Saleh D: Reduced expression of endothelial nitric oxide synthase in the lungs of patients with pulmonary hypertension. N Engl J Med 1995;333:214–221.

4 Cremona G, Higenbottam T, Borland C, Mist B: Mixed expired nitric oxide in primary pulmonary hypertension in relation to lung diffusion capacity. Q J Med 1994;87:547–551.

5 Kaneko F, Arroliga A, Dweik R, Comhair S, Laskowski D, Oppedisano R, Thomassen M, Erzurum SC: Biochemical reaction products of nitric oxide as quantitative markers of primary pulmonary hypertension. Am J Respir Crit Care Med 1998;158:917–923.

6 Archer SL, Djaballah K, Humbert M, Weir KE, Fartoukh M, Dall'ava-Santucci J, Mercier JC, Simonneau G, Dinh-Xuan, AT: Nitric oxide deficiency in fenfluramine- and dexfenfluramine-induced pulmonary hypertension. Am J Respir Crit Care Med 1998;158:1061–1067.

7 Forte P, Copland M, Smith LM, Milne E, Sutherland J, Benjamin N: Basal nitric oxide synthesis in essential hypertension. Lancet 1997;349:837–842.

8 Tsikas D, Gutzki F, Rossa S, Bauer H, Neumann C, Dockendorff K, Sandmann J, Frolich JC: Measurement of nitrite and nitrate in biological fluids by gas chromatography-mass spectrometry and by the Griess assay: Problems with the Griess assay – Solutions by gas chromatography-mass spectrometry. Anal Biochem 1997;244:208–220.

9 Dhein S, Salameh A, Berkels R, Klaus W: Dual mode of action of dihydropyridine calcium antagonists. Drugs 1999;58:397–404.

10 Ding Y, Vaziri N: Nifedipine and diltiazem but not verapamil up-regulate endothelial nitric oxide synthase expression. J Pharmacol Exp Ther 2000;292:606–609.

11 Pearson D, Dawling S, Walsh W, Haines J, Christman B, Bazyk A, Scott N, Summar ML: Neonatal pulmonary hypertension – Urea-cycle intermediates, nitric oxide production, and carbamoyl-phosphate synthetase function. N Engl J Med 2001;344:1832–1838.

12 Gorenflo M, Zheng C, Werle E, Fiehn W, Ulmer HE: Plasma levels of asymmetrical dimethyl-L-arginine in patients with congenital heart disease and pulmonary hypertension. J Cardiovasc Pharmacol 2001;37:489–492.

13 Cracowski JL, Cracowski C, Bessard G, Pepin JL, Bessard J, Schwebel C, Stanke-Labesque F, Pison C: Increased lipid peroxidation in patients with pulmonary hypertension. Am J Respir Crit Care Med 2001;164:1038–1082.

14 Bowers R, Cool C, Murphy RC, Tuder RM, Hopken MW, Flores SC, Voelkel NF: Oxidative stress in severe pulmonary hypertension. Am J Respir Crit Care Med 2004;169:764–769.

15 Wennmalm A, Benthin G, Edlund A, Kieler-Jensen N, Lundin S, Petersson A, Waagstein F: Nitric oxide synthesis and metabolism in man. Ann NY Acad Sci 1994;714:158–164.

16 Demoncheaux EAG, Higenbottam TW, Foster PJ, Borland CDR, Smith APL, Marriott HM, Bee D, Akamine S, Davies MB: Circulating nitrite anions are a directly acting vasodilator and are donors for nitric oxide. Clin Sci 2002;102:77–83.

17 Eiserich J, Baldus S, Brennan M, Ma W, Zhang C, Tousson A, Castro L, Lusis AJ, Nauseef WM, White CR, Freeman BA: Myeloperoxidase, a leukocyte-derived vascular NO oxidase. Science 2002;296:2391–2394.

18 Cosby K, Partovi KS, Crawford JH, Patel RP, Reiter CD, Martyr S, Yang BK, Waclawiw MA, Zalos G, Xu X: Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. Nat Med 2003;9:1498–1505.