Increased Nitric Oxide Production Accompanies Blunted Hypoxic Pulmonary Vasoconstriction in Hyperoxic Rat Lung

Diana Yu-Wung Yeh[1], Shang Jyh Kao[1], Nan Hsiung Feng[2], Hsing I Chen[3], and David Wang[4]

[1]*Division of Chest Medicine*
Department of Internal Medicine
Shin Kong Wu-Ho-Su Memorial Hospital, Taipei
[2]*Department of Internal Medicine*
Kaohsiung Military General Hospital, Kaohsiung
[3]*Institute of Medical Sciences*
Tzu Chi University, Hualien
and
[4]*Department of Medicine, College of Medicine*
Fu Jen Catholic University
Taipei, Taiwan, ROC

Abstract

Hyperoxia may affect lung physiology in different ways. We investigated the effect of hyperoxia on the protein expression of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS), nitric oxide (NO) production, and hypoxic pulmonary vasoconstriction (HPV) in rat lung. Twenty-four male rats were divided into hyperoxic and normoxic groups. Hyperoxic rats were placed in >90% F_IO_2 for 60 h prior to experiments. After baseline *in vitro* analysis, the rats underwent isolated, perfused lung experiments. Two consecutive hypoxic challenges (10 min each) were administered with the administration of a non-specific NOS inhibitor, N-nitro-$_L$-arginine methyl ester (L-NAME), in between. We measured intravascular NO production, pulmonary arterial pressure, and protein expression of eNOS and iNOS by immunohistochemistry. We found that hyperoxia rats exhibited increased baseline NO production ($P < 0.001$) and blunted HPV response ($P < 0.001$) during hypoxic challenges compared to normoxia rats. We also detected a temporal association between the attenuation in HPV and increased NO production level with a negative pre-L-NAME correlation between HPV and NO ($R = 0.52$, $P < 0.05$). After L-NAME administration, a second hypoxic challenge restored the HPV response in the hyperoxic group. There were increased protein expression of eNOS (12.6 ±3.1-fold, n = 3) (X200) and iNOS (8.1 ± 2.6-fold, $n = 3$) (X200) in the hyperoxia group. We conclude that hyperoxia increases the protein expression of eNOS and iNOS with a subsequent increased release of endogenous NO, which attenuates the HPV response.

Key Words: nitric oxide synthase; hyperoxia; hypoxic pulmonary vasoconstriction; nitric oxide; isolated lung

Introduction

Hyperoxia is often used to treat hypoxic pulmonary conditions in clinical practice. Prolonged hyperoxia, however, may be harmful, often leading to pulmonary fibrosis if the patient recovers from the initial insult. There are perhaps different ways through which hyperoxia can affect the lung (21, 25). One

Corresponding author: Dr. David Wang, Department of Medicine, College of Medicine, Fu Jen Catholic University, 510 Chung Cheng Rd., HsinChuang, Taipei 24205, Taiwan, ROC. Tel: 886-2-29053489, Fax: 886-2-29052096, E-mail: m004917@ms.skh.org.tw
Received: November 25, 2005; Revised (Finally): April 26, 2006; Accepted: May 17, 2006.

mechanism relates to the formation of oxygen radical species (7). Oxygen level itself, however, may also affect pulmonary vasoreactivity through as yet unclear mechanisms. Nitric oxide (NO) is a potent vasodilator and has been implicated in the hypoxic pulmonary vasoconstriction (HPV) response (29). There are reports that showed that hyperoxia may induce nitric oxide production in the lung (4, 30).

HPV is a well-known phenomenon since its first description by von Euler and Liljestrand in 1946 (36). Although it may help to temporarily offset ventilation/perfusion ($\dot{V}/\dot{Q}$) mismatch in some clinical situations, sustained HPV may lead to pulmonary hypertension, vascular remodeling, and cor pulmonale. The mechanisms behind the HPV response are likely complex and multiple. Leach *et al*. demonstrated biphasic contraction of pulmonary vessels undergoing hypoxic insult, which implies multi-step regulation (18). Impaired HPV response is known to play an important role in certain clinical situations such as chronic obstructive pulmonary disease and high altitude pulmonary edema (10, 26). NO is one of the many possible mediators responsible for HPV. We know that chronic hypoxia down-regulates the HPV response with up-regulation of both endothelial and inducible nitric oxide synthases (eNOS and iNOS) (31, 38). An understanding of the mechanism behind hypoxic vasoconstriction may lead to future development of adjunct therapies for diseases such as acute respiratory distress syndrome, high-altitude sickness, and chronic obstructive pulmonary disease.

In this study we test the hypothesis that hyperoxia induces NO production, which in turn attenuates the HPV response.

Materials and Methods

Environmental Chambers

Male Sprague-Dawley rats weighing 300 to 350 g were placed in room air (normoxia group) or a hyperoxic environment (> 90% F_IO_2) (hyperoxia group) for 60 h prior to experiments. Each rat was housed in an individual 20 liter Plexiglas chamber with adequate food and water supply. Chambers contained soda lime to maintain an environmental pCO_2 < 2 mm Hg, verified 2 to 3 times daily. An oxygen flow rate of 6 to 8 liters per minute was used in hyperoxic chambers. F_IO_2 was monitored continuously with an O_2 sensor. Rats in the room air group had wire mesh instead of solid Plexiglas doors in their chambers. The chambers were cleaned once daily, and each hyperoxia rat's exposure to room air was less than 5 min each time. All experimental procedures were approved by the University Animal Care and Use Committee.

Isolation and Perfusion of Rat Lungs

The lungs of anesthetized (pentobarbital 50 mg/kg, ip) and tracheostomized Sprague-Dawley rats (300-350g) were isolated and perfused *in situ* as previously described (14). A cannula (inflow) and a large catheter (outflow) were inserted into the pulmonary artery and the left atrium, respectively. In addition to the inflow and outflow cannulae, the perfusion system consisted of a reservoir and a roller pump. The lungs were artificially ventilated and perfused *via* the roller pump at a constant flow (8-10 ml/min) with a physiological saline solution (PSS; in mM: 116.3 NaCl, 5.4 KCl, 0.83 $MgSO_4$, 19.0 $NaHCO_3$, 1.04 NaH_2PO_4, 1.8 $CaCl_2$. H_2O, 5.5 D-glucose, pH 7.4 mixed with 4% bovine serum albumin and 3 U/ml heparin). The venous outflow from the left atrium was collected into the reservoir. A water bath was used to pre-warm the PSS and kept at a constant temperature (37 ± 0.5°C). The volume inside the perfusion system was kept at 15 ml. The pulmonary arterial pressure (PAP) was measured with a pressure transducer (Gould Instrument, Cleveland, OH, USA) connected to a side arm from the inflow cannula.

Measurement of Nitric Oxide Production from Isolated Lungs

NO was measured with a commercially available NO meter (model NO 501 monitoring device, Int. Co., Nagoya, Japan). The NO monitoring device consisted of an ammeter with a built-in power supply and electrodes for the detection of NO. The electrodes included a working electrode and a carbon fiber counter-electrode. The working electrode was placed through a side arm connecting to the outflow catheter draining the pulmonary venous fluid, and the counter-electrode was fixed to touch the surface of the lung tissue (37). Once the basic current became stable, the NO response current was continuously recorded. The peak response was noted for the measurement of the maximal NO production. In addition to the original perfusion system to the isolated lungs, an additional perfusion system including a pump and a side-tubing was introduced between the inflow cannula and the NO working electrode. The system bypassing the lungs was designed to measure the basal release of NO in the perfusate without coursing through the pulmonary circuit. At the same time, the perfusing system for the isolated lungs was kept running without interruption. After a period of NO measurement from the bypass, the additional perfusion circuit was stopped and switched to NO measurement from the outflow of the pulmonary circulation. S-nitroso-N-acetyl-DL-penicillamine (SNAP) was used as a standard to calculate the NO production. One mM of SNAP

produces 1 μM of NO and shows a 200 pA increase on the NO monitor.

In Vitro Measurement of Baseline NO Release from Blood

For measurement of the NO release from the blood obtained from normoxia rats and hyperoxia rats, the same NO meter (Model NO-501, Inter Medical Co., Nagoya, Japan) consisting of a working electrode and a counter-electrode (11) was used. The electrode pair was immersed in a small chamber containing 3 ml of Krebs buffer (pH 7.4) with 0.05 M arginine. The two electrodes were positioned within 10 mm of each other and the solution in the chamber was stirred by a magnetic stirrer. After the basic current became stable, 200 μl of heparinized blood obtained from normoxia rats and hyperoxia rats were added to the chamber, and peak NO response current curves were obtained for comparison.

Immunohistochemistry

Lung tissues were dissected after 60 h of hyperoxia for immunochemical analysis of the protein expressions of eNOS and iNOS. Lung tissues from hyperoxic (n = 3) and control rats (n = 3) were fixed in tissue fix buffer, embedded in Super-Tek OCT compound (Gene Research Laboratory, Taipei, Taiwan, catalog numbers: PS0001 and PS0002), and frozen in liquid nitrogen. Sections were cut on a cryostat (Leica CM1900, Nussloch, Germany), then thawed and mounted onto gelatin-coated slides. All 5 μm frozen lung sections from the hyperoxic and control groups were used for immunohistochemical staining.

Lung sections were first incubated with a blocking reagent, then with the appropriate dilution of the primary antibody (mouse anti-rat eNOS or anti-rat iNOS monoclonal antibody at a titer of 1:50; Chemicon MAb, 13421, Temecula, CA, USA), and finally with an anti-mouse IgG-horseradish peroxidase (HRP) secondary antibody at a titer of 1:100. Sections were labeled and developed with HRP substrate solution and counterstained with a hematoxylin stain kit (PS003, Gene Research Laboratory, Taipei, Taiwan). To quantify immunohistochemical differences in rat lung sections without relying on subjective assessments, we used digital imaging and the Image-Pro Plus (Media Cybernetics, Silver Spring, MD, USA) microimaging package. Data were collected and analyzed by the method described in the user guide in the Counting, Measuring and Classifying sections. The overall fields from each section of each tissue were digitally captured with a high-resolution cooled CCD camera (ProgRes C14, Jenoptik Laser, Optik, System GmbH, Jena, Germany) and stored as 8-bit color images. The immunostained tissue cells were automatically highlighted by Image-Pro Plus; the area covered by immunohistochemically positive cells (with red color) was scored as positive and divided by the total area.

Experimental Protocol

Part 1: Baseline NO release from the blood of hyperoxia and normoxia rats

Two hundred μl of each blood sample of both the hyperoxic (n = 9) and the normoxic (control) (n = 9) groups were added to Kreb's buffer with 0.05 M arginine. NO release was measured for 7 min until the curve plateaued.

Part 2: Consecutive challenges with 5% CO_2 - 95% N_2 gas mixture

Lungs from 9 hyperoxia rats and 9 normoxia rats underwent two consecutive hypoxic challenges. The isolated lungs were ventilated with 21% O_2 - 5% CO_2 - 74% N_2 humidified mixture at the beginning of the experiment. Changes in NO concentration and the PAP were recorded continuously throughout the experiment. After the PAP has stabilized, the first hypoxic challenge was administered at time 0 min. The lungs were ventilated with 5% CO_2 - 95% N_2 gas mixture for 10 min. Data were graphed at times 0, 1, 2, 3, 5, 7, 9, and 10 min. Once the PAP and the NO response curve had returned to baseline after conclusion of the first hypoxic challenge, L-NAME was added to the reservoir (1 mM) and the system allowed to equilibrate for 20 min. Data were graphed at times 12.5, 15, 20 (reoxygenation) and 30, 35, 37.5 (stabilization) min. A second hypoxic challenge was then administered for 10 min (data points at 40, 41, 42, 43, 45, 47, 49 min) and the system allowed to reoxygenate again (data points at 50, 52.5, 55, and 60 min). The amount of time separating the 2 challenges was determined based on the speed of recovery after the first challenge.

Part 3: Immunohistochemistry analysis

Lung tissues were obtained from normoxia rats (n = 3) and hyperoxia rats (n = 3) for immunohistochemical staining.

Statistical Analysis

Data were expressed as means ± SEM. The difference between groups was evaluated by Student's *t*-test. A *P* value less than 0.05 was considered statistically significant.

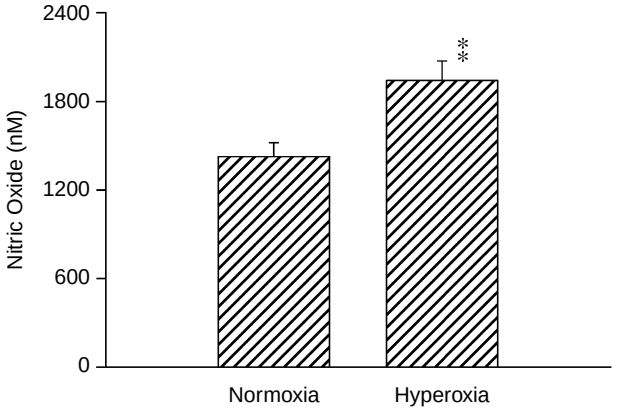

Fig. 1. Whole blood obtained from hyperoxia rats yields more nitric oxide when compared to normoxia (room air) rats (**, $P < 0.01$).

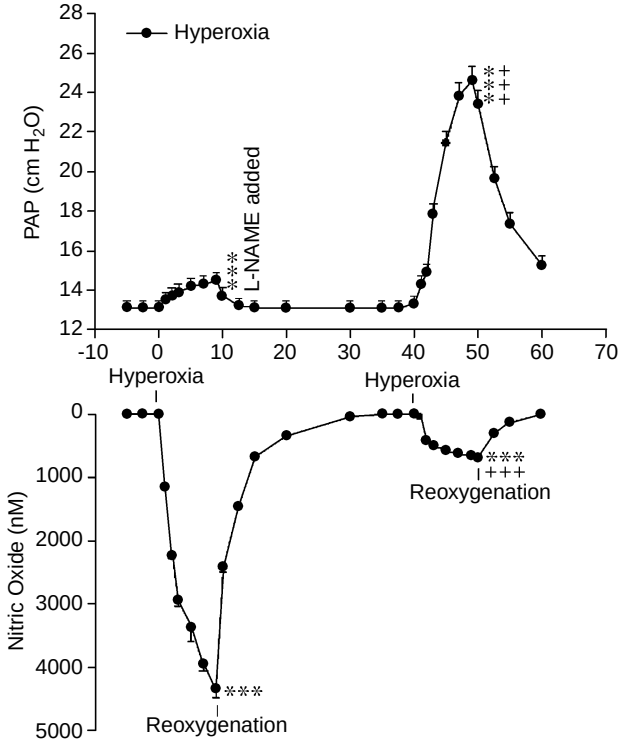

Fig. 2. Hypoxia induced a big release of nitric oxide (***, $P < 0.001$ *vs.* baseline) and a concomitant attenuation of the hypoxic pulmonary vasoconstriction response in hyperoxia rats. After adding a non-specific NOS inhibitor, N-nitro-$_L$-arginine methyl ester (L-NAME), NO release was significantly attenuated (+++, $P < 0.001$), and the PAP response was significantly potentiated (+++, $P < 0.001$). The ratio of the post-ΔPAP/ pre-ΔPAP showed an 11-fold increase.

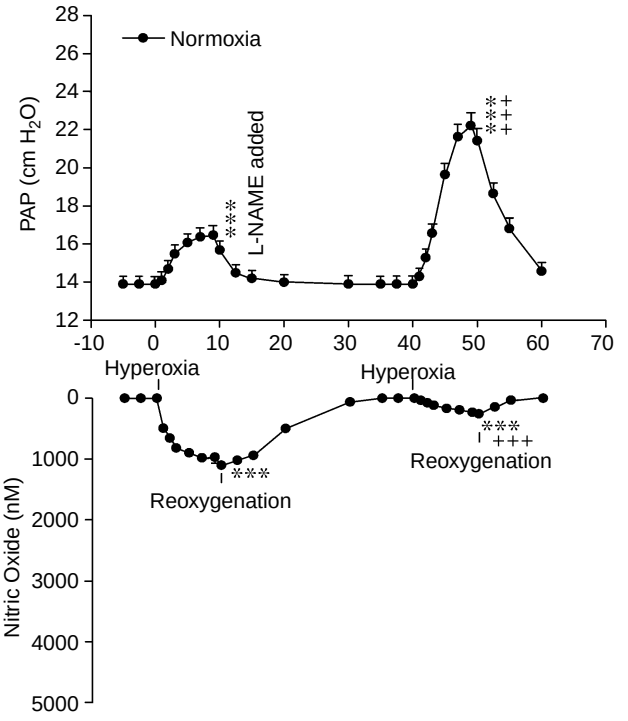

Fig. 3. Hypoxia induced a more marked hypoxic pulmonary vasoconstriction response ($P < 0.001$) and a less prominent release of nitric oxide ($P < 0.001$) in normoxia rats. After adding N-nitro-$_L$-arginine methyl ester (L-NAME), NO release was attenuated (+++, $P < 0.001$), and the PAP response increased (+++, $P < 0.001$). The ratio of post-ΔPAP/ pre-ΔPAP was increased 3-fold.

Results

In the *in vitro* part of the experiment, the blood of hyperoxia rats produced significantly more NO compared to the blood of normoxia rats. The peak values for the NO responses of the hyperoxia and normoxia rats were 1942 ± 132 nM and 1426 ±95 nM, respectively ($P < 0.01$) (Fig. 1).

In the second part of the experiment, hypoxia induced a small rise in the PAP and a concomitant burst of NO release in the hyperoxic, isolated rat lung. After a non-specific NOS inhibitor, $_L$-NAME, was administered, a second hypoxic challenge induced a higher rise in the PAP and significantly less NO release compared to the first hypoxic challenge (+++, $P < 0.001$) (Fig. 2). In the normoxia rat, hypoxia induced a higher initial rise of PAP and less NO release when compared to the hyperoxic rat (Fig. 3). The baseline PAPs between the two groups are not statistically different. The pre-L-NAME data in both groups showed a negative linear correlation between HPV and NO ($R = 0.52$, $P < 0.05$), while no significant correlation was seen between HPV and NO in post-L-NAME data. In comparison of the baseline NO release between the two groups, we see a near 6-fold increase in the hyperoxia rats. Before and after the addition of L-NAME, the hyperoxia group showed an

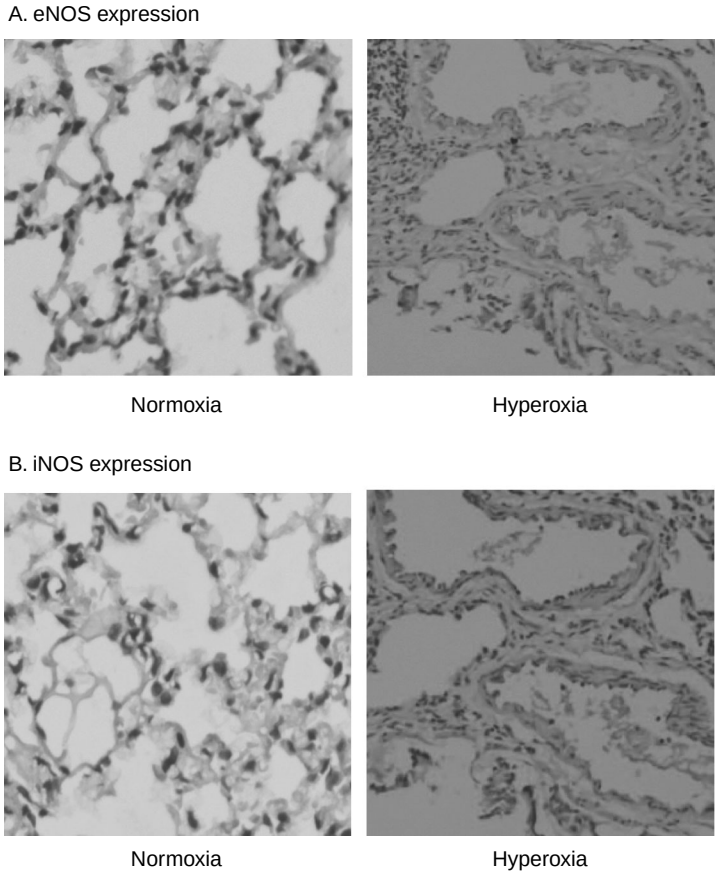

Fig. 4. Immunohistochemical staining of eNOS (A) and iNOS (B) activity in lung tissues of normoxic and hyperoxic groups. Lung tissues were dissected 60 h after hyperoxia for immunohistochemical analysis of protein expressions. In the normoxic group eNOS and iNOS immunohistochemical staining was low. In contrast, the hyperoxic group showed a marked increase in eNOS (X200) ($P < 0.001$) and iNOS (X200) ($P < 0.001$) expressions.

11-fold increase in the post-ΔPAP/ pre-ΔPAP ratio, whereas the normoxia group showed only a 3-fold increase.

In the third part of experiment, examination of the immunohistochemical staining of eNOS and iNOS (n = 3) in rat lung tissue revealed a marked increase in the expression of eNOS (12.6 ± 3.1-fold, n = 3; $P < 0.001$) and iNOS (8.1 ± 2.6-fold, n = 3, $P < 0.001$) in the hyperoxic group compared with the normoxic group (Fig. 4).

Discussion

Hyperoxia is an often-encountered clinical problem during the treatment of hypoxic pulmonary conditions. It has several effects on the lung. One of which is the production of oxygen free radicals. Is has long been known that oxygen free radicals can cause lung tissue injury (16, 17, 35). Another effect of hyperoxia on the lung relates to surfactant secretion. Hyperoxia impairs surfactant production, which may also contribute to lung injury (42). It has been reported that a brief period (20 h) of hyperoxia causes decreased surfactant activity in premature rabbits (13). Despite what is known about the detrimental effects of hyperoxia, it is often unavoidable in many clinical situations. Hyperoxia has applications in treating decompression sickness, slow-healing decubitus or diabetic ulcers, chronic osteomyelitis, burns, and hypoxic conditions such as pneumonia and the acute respiratory distress syndrome. In view of its extensive application, what we can learn about its effects on lung tissue may one day become relevant clinically.

Another significant effect of hyperoxia on the lung with clinical implications is the blunting of the HPV response (23, 24, 34). Thus far two broad categories of mechanisms have been proposed for the HPV response. One involves endogenous mediators with vasoactive properties. The other involves calcium or potassium channels in the smooth muscle cell of the pulmonary vasculature. With regards to the mediator hypothesis, there are many proposed mediators. Prostaglandin products produced by the cyclooxygenase pathway and NO all have vasodilating effects and

may each be the agent(s) responsible for reducing the HPV response in hyperoxia. Leeman *et al*. reported in 1999 that the magnitude of the HPV response is affected by the co-release of cyclooxygenase metabolites and of NO (19). Other mediators/modulators implicated include activated protein kinase-C, products of the superoxide-hydrogen peroxide axis, and NO-dependent guanylate cyclase (39-41). With regards to NO, there is thus far only indirect evidence that NO may be the responsible agent. Nitric oxide (NO) is an important vasodilator for regulating the basal vessel tone. It is also highly reactive and therefore short-lived under physiological condition. Maggiorini *et al*. (20) reported that inhaled NO reversed the hypoxia-induced changes in pulmonary vessel impedance in both dogs and pigs. Romand *et al*. (28) also noted partial reversal of hypoxia-induced increases in pulmonary pressure after NO inhalation in anesthetized dogs. These studies showed only the effect of exogenous NO. They noted no effect of NO inhalation on pulmonary pressure during hyperoxia, however. With regards to endogenous NO, we know that the inhibition of endogenous NO can increase or restore the HPV response (1, 3, 5, 6, 12). Robertson *et al*. (27) found that inhibition of nitric oxide synthesis in isolated rat lung increased the vasoconstriction response in hypoxia, but they did not observe the same effect in hyperoxia. Suzuki and coworkers (33) noted that blunted HPV in hyperoxia was restored by the inhibition of eNOS , but not by the inhibition of iNOS or cyclooxygenase. This is in contrast to our result where we found that both eNOS and iNOS expression was enhanced in the immunohistochemical staining of hyperoxia rat lung. In the Suzuki *et al*. study, however, only pulmonary arterial pressure and pulmonary vessel diameter were measured, without quantification of the intravascular NO content as we performed in our study. One report by Arkovitz *et al*. (2) noted that hyperoxia increased NO production as measured by nitrate and nitrite concentration in the broncheoalveolar lavage fluid. The same increase, however, was not noted in the serum in their work. Another report by Steudel *et al*. (32) documented increased nitric oxide synthase (NOS) expression in vascular endothelial cells and in alveolar macrophages in hyperoxia. In our study, we were able to detect an increased NO production temporally associated with blunted HPV response after hyperoxia. A negative correlation was also observed between HPV and NO production in the pre- L-NAME data. The fact that the same was not seen in the post- L-NAME data may have been due to variable intensity of response to L-NAME among individual rats which, when pooled, obscure the correlation. After L-NAME administration, NO production was higher in hyperoxia rats compared to normoxia rats. The seemingly paradoxical observation in NO production may be explained by the higher existing (basal) level of NO in hyperoxia rats and the possibility of incomplete inhibition of NO synthases by L-NAME due to insufficient dosage. PAP levels after L-NAME administration cannot be compared between groups due to variable individual response to L-NAME. However, intra-group showed an exaggerated response in pre- and post- L-NAME PAP in the hyperoxia group when compared to the normoxia group. Immunohistochemistry analysis in our study also pointed to increased expression of both eNOS and iNOS in hyperoxia rat lungs. These observations provide strong evidence that hyperoxia induces a significant amount of NO production from rat lung mediated probably through both iNOS and eNOS, which attenuates the HPV response.

What are the effects of NO on lung tissue, apart from its role in HPV? If we know that NO is one of the mediators involved in HPV, can we use this information to develop treatment or prevention strategy for the undesirable consequences of sustained HPV? NO is known as a chameleon with both pro-oxidant and anti-oxidant activities (9). There are conflicting reports regarding its impact on hyperoxic injury. Inhibition of the endogenous production of NO was observed to worsen hyperoxic lung damage by measuring endothelial and alveolar permeability, lung liquid clearance, and lung weight by Garat *et al*. (8). On the contrary, intermittent exposure to NO was noted to exert a synergistic cytotoxic effect with hyperoxia on cultured lung cells in work by Narula *et al*. (22). Similar to hyperoxia, NO also has an effect on surfactant production in the lung. Issa observed that a low dosage of inhaled NO decreased hyperoxia-induced detrimental effects on alveolar surfactant production (13). However, when reacted upon by superoxide radicals, NO forms peroxynitrite, a substance which can decrease surfactant activity (9). Another observation regarding NO is that inhaled NO can redistribute pulmonary perfusion, resulting in a general increased perfusion in all regions of the lung (14). This may be related to the blunting of the HPV response by NO. Based on these conflicting reports, it is conceivable that NO may play a regulatory role in the lung, having both a detrimental and a beneficial effect, depending on the physiological condition. These effects of NO on the lung apart from the HPV response, however, were not within the scope of the present study and are subjects for future investigation. By elucidating further the role of NO in the lung, we may hope to one day develop strategies in treating hyperoxia- or hypoxia-related lung conditions in the future.

In conclusion, we demonstrated in our study that hyperoxia induces increased nitric oxide production by direct detection of nitric oxide both *in vitro* and in an isolated, perfused rat lung model. This

increased production is mediated by NOS as detected by increased protein expressions of eNOS and iNOS by immunohistochemistry. We also showed that hyperoxia-related attenuation of the HPV response is accompanied by an increased NO level with a close temporal correlation. This indicates to us that NO may play an important role in both hyperoxic lung conditions and the attenuation of the HPV response.

References

1. Archer, S.L., Tolins, J.P., Raij, L. and Weir, E.K. Hypoxic pulmonary vasoconstriction is enhanced by inhibition of the synthesis of an endothelium-derived relaxing factor. *Biochem. Biophys. Res. Commun*. 164: 1198-1205, 1989.
2. Arkovitz, M.S., Szabo, C., Garcia, V.F., Wong, H.R. and Wispe, J.R. Differential effects of hyperoxia on the inducible and constitutive isoforms of nitric oxide synthase in the lung. *Shock* 7: 345-350, 1997.
3. Bardou, M., Goirand, F., Marchand, S., Rouget, C., Devillier, P., Dumas, J.P., Morcillo, E.J., Rochette, L. and Dumas, M. Hypoxic vasoconstriction of rat main pulmonary artery: role of endogenous nitric oxide, potassium channels, and phosphodiesterase inhibition. *J. Cardiovasc. Pharmacol*. 38: 325-334, 2001.
4. Comhair, S.A., Thomassen, M.J. and Erzurum, S.C. Differential induction of extracellular glutathione peroxidase and nitric oxide synthase 2 in airways of healthy individuals exposed to 100% O_2 or cigarette smoke. *Am. J. Respir. Cell Mol. Biol*. 23: 350-354, 2000.
5. Emery, C.J., Teng, G.Q., Liu, X. and Barer, G.R. Vasoreactions to acute hypoxia, whole lungs and isolated vessels compared: modulation by NO. *Respir. Physiol. Neurobiol*. 134: 115-129, 2003.
6. Ferrario, L., Amin, H.M., Sugimori, K., Camporesi, E.M. and Hakim, T.S. Site of action of endogenous nitric oxide on pulmonary vasculature in rats. *Pflugers Arch*. 432: 523-527, 1996.
7. Freeman, B.A. and Crapo, J.D. Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J. Biol. Chem*. 256: 10986-10992, 1981.
8. Garat, C., Jayr, C., Eddahibi, S., Laffon, M., Meignan, M. and Adnot, S. Effects of inhaled nitric oxide or inhibition of endogenous nitric oxide formation on hyperoxic lung injury. *Am. J. Respir. Crit. Care Med*. 155: 1957-1964, 1997.
9. Hallman, M. and Bry, K. Nitric oxide and lung surfactant. *Semin. Perinatol*. 20: 173-185, 1996.
10. Hultgren, H.N. High altitude pulmonary edema: hemodynamic aspects. *Int. J. Sports Med.* 18: 20-25, 1997.
11. Ichimori, K., Ishida, H., Fukahori, M., Nakazawa, H. and Murakam, E. Practical nitric oxide measurement employing a nitric oxide-selective electrode. *Rev. Sci. Instrum*. 65: 2714-2718, 1994.
12. Ignarro, L.J., Byrns, R.E., Buga, G.M., Wood, K.S. and Chaudhuri, G. Pharmacological evidence that endothelium-derived relaxing factor is nitric oxide: use of pyrogallol and superoxide dismutase to study endothelium-dependent and nitric oxide-elicited vascular smooth muscle relaxation. *J. Pharmacol. Exp. Ther*. 244: 181-189, 1988.
13. Issa, A., Lappalainen, U., Kleinman, M., Bry, K. and Hallman, M. Inhaled nitric oxide decreases hyperoxia-induced surfactant abnormality in preterm rabbits. *Pediatr. Res*. 45: 247-254, 1999.
14. Jones, A.T., Hansell, D.M. and Evans, T.W. Pulmonary perfusion quantified by electron-beam computed tomography: effects of hypoxia and inhaled NO. *Eur. Respir. J*. 21: 855-861, 2003.
15. Kao, S.J., Wang, D., Yeh, D.Y., Hsu, K., Hsu, Y.H. and Chen, H.I. Static inflation attenuates ischemia/reperfusion injury in an isolated rat lung *in situ*. *Chest* 126: 552-558, 2004.
16. Kelly, F.J. and Lubec, G. Hyperoxic injury of immature guinea pig lung is mediated *via* hydroxyl radicals. *Pediatr. Res.* 38: 286-291, 1995.
17. Kinnula, V.L. and Crapo, J.D. Superoxide dismutases in the lung and human lung diseases. *Am. J. Respir. Crit. Care Med*. 167: 1600-1619, 2003.
18. Leach, R.M., Robertson, T.P., Twort, C.H. and Ward, J.P. Hypoxic vasoconstriction in rat pulmonary and mesenteric arteries. *Am. J. Physiol*. 266: L223-L231, 1994.
19. Leeman, M., de Beyl, V.Z., Biarent, D., Maggiorini, M., Melot, C. and Naeije, R. Inhibition of cyclooxygenase and nitric oxide synthase in hypoxic vasoconstriction and oleic acid-induced lung injury. *Am. J. Respir. Crit. Care Med*. 159: 1383-1390, 1999.
20. Maggiorini, M., Brimioulle, S., De Canniere, D., Delcroix, M., Wauthy, P. and Naeije, R. Pulmonary vascular impedance response to hypoxia in dogs and minipigs: effects of inhaled nitric oxide. *J. Appl. Physiol*. 79: 1156-1162, 1995.
21. Mantell, L.L. and Lee, P.J. Signal transduction pathways in hyperoxia-induced lung cell death. *Mol. Genet. Metab*. 71: 359-370, 2000.
22. Narula, P., Xu, J., Kazzaz, J.A., Robbins, C.G., Davis, J.M. and Horowitz, S. Synergistic cytotoxicity from nitric oxide and hyperoxia in cultured lung cells. *Am. J. Physiol*. 274: L411-L416, 1998.
23. Newman, J.H., Loyd, J.E., English, D.K., Ogletree, M.L., Fulkerson, W.J. and Brigham, K.L. Effects of 100% oxygen on lung vascular function in awake sheep. *J. Appl. Physiol*. 54: 1379-1386, 1983.
24. Newman, J.H., McMurtry, I.F. and Reeve, J.T. Blunted pulmonary pressor responses to hypoxia in blood perfused, ventilated lungs isolated from oxygen toxic rats: possible role of prostaglandins. *Prostaglandins* 22: 11-20, 1981.
25. Pagano, A. and Barazzone-Argiroffo, C. Alveolar cell death in hyperoxia-induced lung injury. *Ann. N.Y. Acad. Sci*. 1010: 405-416, 2003.
26. Peinado, V.I., Santos, S., Ramirez, J, Roca, J., Rodriguez-Roisin, R. and Barbera, J.A. Response to hypoxia of pulmonary arteries in chronic obstructive pulmonary disease: an *in vitro* study. *Eur. Respir. J*. 20: 332-338, 2002.
27. Robertson, B.E., Warren, J.B. and Nye, P.C. Inhibition of nitric oxide synthesis potentiates hypoxic vasoconstriction in isolated rat lungs. *Exp. Physiol*. 75: 255-257, 1990.
28. Romand, J.A., Pinsky, M.R., Firestone, L., Zar, H.A. and Lancaster, J.R. Jr. Inhaled nitric oxide partially reverses hypoxic pulmonary vasoconstriction in the dog. *J. Appl. Physiol.* 76: 1350-1355, 1994.
29. Rumsey, W.L., Abbott, B.M., Bertelsen, D.L. and DeSiato, M.A. Nitric oxide and endothelin in acute hypoxic pulmonary vasoconstriction and adaptation to chronic hypoxia. *Adv. Exp. Med. Biol.* 428: 173-180, 1997.
30. Schmetterer, L., Strenn, K., Kastner, J., Eichler, H.G. and Wolzt, M. Exhaled NO during graded changes in inhaled oxygen in man. *Thorax* 52: 736-738, 1997.
31. Shirai, M., Pearson, J.T., Shimouchi, A., Nagaya, N., Tsuchimochi, H., Ninomiya, I. and Mori, H. Changes in functional and histological distributions of nitric oxide synthase caused by chronic hypoxia in rat small pulmonary arteries. *Br. J. Pharmacol*. 139: 899-910, 2003.
32. Steudel, W., Watanabe, M., Dikranian, K., Jacobson, M. and Jones, R.C. Expression of nitric oxide synthase isoforms (NOS II and NOS III) in adult rat lung in hyperoxic pulmonary hypertension. *Cell Tissue Res.* 295: 317-329, 1999.
33. Suzuki, K., Naoki, K., Kudo, H., Nishio, K., Sato, N., Aoki, T., Suzuki, Y., Takeshita, K., Miyata, A., Tsumura, H., Yamakawa, Y. and Yamaguchi, K. Impaired hypoxic vasoconstriction in intraacinar microvasculature in hyperoxia-exposed rat lungs. *Am. J. Respir. Crit. Care Med*. 158: 602-609, 1998.
34. Troug, W.E., Redding, G.J. and Stadert, T.A. Effects of hyperoxia on vasoconstriction and VA.Q matching in the neonatal lung. *J. Appl. Physiol*. 63: 2536-2541, 1987.
35. Tsan, M.F. Superoxide dismutase and pulmonary oxygen toxicity: lessons from transgenic and knockout mice. *Int. J. Mol. Med.* 7: 13-

19, 2001.
36. Von Euler, U.S. and Liljestrand, G. Observations on the pulmonary arterial blood pressure in the cat. *Acta Physiol. Scand*. 12: 301-320, 1946.
37. Wang, D., Hsu, K., Hwang, C.P. and Chen, H.I. Measurement of nitric oxide release in the isolated perfused rat lung. *Biochem. Biophys. Res. Commun*. 208: 1016-1020, 1995.
38. Weissmann, N., Nollen, M., Gerigk, B., Ardeschir Ghofrani, H., Schermuly, R.T., Gunther, A., Quanz, K., Fink, L., Hanze, J., Rose, F., Seeger, W. and Grimminger, F. Downregulation of hypoxic vasoconstriction by chronic hypoxia in rabbits: effects of nitric oxide. *Am. J. Physiol.* 284: H931-H938, 2003.
39. Weissmann, N., Voswinckel, R., Hardebusch, T., Rosseau, S., Ghofrani, H.A., Schermuly, R., Seeger, W. and Grimminger, F. Evidence for a role of protein kinase C in hypoxic pulmonary vasoconstriction. *Am. J. Physiol*. 276: L90-L95, 1999.
40. Weissmann, N., Voswinckel, R., Tadic, A., Hardebusch, T., Ghofrani, H.A., Schermuly, R.T., Seeger, W. and Grimminger, F. Nitric oxide (NO)-dependent but not NO-independent guanylate cyclase activation attenuates hypoxic vasoconstriction in rabbit lungs. *Am. J. Respir. Cell Mol. Biol*. 23: 222-227, 2000.
41. Weissmann, N., Winterhalder, S., Nollen, M., Voswinckel, R., Quanz, K., Ghofrani, H.A., Schermuly, R.T., Seeger, W. and Grimminger, F. NO and reactive oxygen species are involved in biphasic hypoxic vasoconstriction of isolated rabbit lungs. *Am. J. Physiol.* 280: L638-L645, 2001.
42. Zenri, H., Rodriquez-Capote, K., McCaig, L., Yao, L.J., Brackenbury, A., Possmayer, F., Veldhuizen, R. and Lewis, J. Hyperoxia exposure impairs surfactant function and metabolism. *Crit. Care Med*. 32: 1155-1160, 2004.