Hypoxia inhibits increased $ET_B$ receptor-mediated NO synthesis in hypertensive rat lungs

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Sato, Koichi, David M. Rodman, and Ivan F. McMurry. Hypoxia inhibits increased $ET_B$ receptor-mediated NO synthesis in hypertensive rat lungs. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol., 20): L511–L581, 1999.—Although hypertensive lungs of chronically hypoxic rats express increased levels of nitric oxide (NO) synthases (NOSs) and produce increased amounts of NO-containing compounds (NOx) during normoxic ventilation, the level of NO production during hypoxic exposure is unclear. Because hypoxia inhibits NO synthesis in normotensive lungs, we investigated whether hypoxic ventilation inhibited NO synthesis in isolated hypertensive lungs and chronically hypoxic rats. Measurement of perfusate NOx concentration in hypertensive lungs from male rats exposed to 4 wk of hypobaric hypoxia showed that basal NOx production was reduced during hypoxic (0% O2) vs. normoxic (21% O2) ventilation. Similarly, plasma NOx concentration was lower in chronically hypoxic rats breathing 10% O2 than in those breathing 21% O2. Hypoxic inhibition of lung NOx production was not prevented by supplementary L-arginine or tetrahydrobiopterin and was not mimicked by inhibition of Ca2+ influx. However, it was mimicked by inhibition of constitutive NOS with N6-monomethyl-L-arginine and chelation of intracellular Ca2+. The endothelin type B-receptor antagonist BQ-788 prevented the increases in NOx production associated with normoxic ventilation in both isolated hypertensive lungs and intact chronically hypoxic rats. These results suggest that a reduced supply of the cosubstrate molecular O2 to NOS counteracts an endothelin-mediated stimulation of NO synthesis in hypertensive pulmonary remodeling. Resta et al. (28) have more recently found increased eNOS protein in small pulmonary arteries but not in veins of chronically hypoxic rats, and Xue and Johns (47) reported that the pulmonary vascular upregulation of eNOS mRNA and protein is temporally correlated with hypertensive vascular remodeling.

Despite this evidence of increased NOS protein and NO production in hypoxia-induced hypertensive rat lungs, it is obvious that the in vivo NO activity is insufficient to prevent pulmonary hypertension. One uncertainty is whether lung and/or pulmonary vascular NO production is actually increased in intact hypoxic rats. Measurements of increased perfusate levels of NOx in isolated hypertensive lungs by Muramatsu et al. (24) and Isaacson et al. (17) were made during normoxic ventilation, and it may be that NO activity is, in fact, inhibited during the in vivo hypoxic exposure. This possibility is supported by numerous studies of NOS enzyme preparations (1, 8, 27), cultured endothelial cells (3, 44), pulmonary arterial rings (18, 32, 38), and normotensive lungs (5, 7, 13, 19, 25) and of exhaled NO in human subjects (8), which indicated that exposure to acute hypoxia inhibits NO synthesis. Furthermore, a low level of endogenous pulmonary NO production in hypoxic rats could explain both the ineffectiveness of chronic treatment with the NOS inhibitor nitro-L-arginine methyl ester in exacerbating pulmonary hypertension (14) and the effectiveness of relatively low levels of chronic inhaled NO in reducing it (20, 33).

Thus the purpose of this study was to examine whether hypoxic ventilation reduced NO synthesis in hypoxia-induced hypertensive lungs and in intact chronically hypoxic rats. Our measurements of perfusate NOx in isolated lungs and of plasma NOx in intact rats indicated that NO production was inhibited during hypoxic ventilation. Additional isolated lung experiments showed that hypoxic inhibition was neither

The levels of lung and pulmonary vascular nitric oxide (NO) production in chronically hypoxic rats are unclear and controversial. Although hypertensive conduit pulmonary arteries isolated from chronically hypoxic rats have impaired endothelium-dependent relaxation (24, 26, 31, 38), most, but not all (9), perfusion studies of isolated hypertensive lungs showed normal or increased NO synthase (NOS) activity as reflected in increased NO-mediated suppression of basal vascular tone (4, 17, 23, 26, 33), responsiveness to endothelium-dependent vasodilators (4, 17, 24, 26, 30, 33), and perfusate levels of NO-containing compounds (NOx) (17, 24). Studies of NOS gene expression in hypoxic hypertensive rat lungs support the functional evidence of increased activity. In rats exposed to hypoxia for 2–4 wk, Shaoul et al. (37) found increased lung tissue levels of both endothelial NOS (eNOS) and neuronal NOS (nNOS) mRNA and protein, and Xue et al. (48) reported increased NOS protein in both endothelial and smooth muscle cells of pulmonary and bronchial blood vessels and in smooth muscle cells of large bronchi. Both groups measured increased L-arginine to L-citrulline conversion in hypertensive lung homogenates. Le Cras et al. (21) subsequently observed increased levels of both eNOS and inducible NOS (iNOS) protein in hypertensive lungs and de novo expression of eNOS in hypertensive pulmonary resistance vessels. Resta et al. (28) have more recently found increased eNOS protein in small pulmonary arteries but not in veins of chronically hypoxic rats, and Xue and Johns (47) reported that the pulmonary vascular upregulation of eNOS mRNA and protein is temporally correlated with hypertensive vascular remodeling.
prevented by supplementation with cosubstrate L-arginine or cofactor tetrahydrobiopterin nor mimicked by Ca^{2+}-free perfusion to attenuate endothelial cell Ca^{2+} influx. However, the increased NO_{x} production during normoxic ventilation was reduced by a chelator of intracellular Ca^{2+} and a nonselective NOS inhibitor but not by a preferential iNOS blocker. Because the vasoactivity of endogenous endothelin (ET)-1 is increased in hypertensive rat lungs (22, 23) and because ET-1 stimulates endothelial NO production via ET_{b} receptors (16), we also tested and found that an ET_{b}-receptor blocker completely prevented the increased normoxic NO_{x} production. Collectively, the results suggested that direct hypoxic inhibition of NOS counteracted an endogenous ET-1-induced, ET_{b} receptor-mediated stimulation of lung and/or pulmonary vascular NO production in chronically hypoxic rats.

METHODS

Animals. Experiments were performed with two groups of adult male Sprague-Dawley rats (300–400 g). The normoxic, pulmonary-normotensive group was kept at Denver’s altitude of 5,280 ft [barometric pressure (P_{b}) = 630 mmHg, inspired O_{2} tension = 122 mmHg]. The chronically hypoxic, pulmonary-hypertensive group was exposed to a simulated altitude of 17,000 ft [P_{b} = 410 mmHg, inspired O_{2} tension = 76 mmHg] for 1, 2, 4, 8, or 12 wk in a hypobaric chamber flushed continuously with room air to prevent accumulation of CO_{2}, NH_{3}, and H_{2}O. Hypobaric exposure was 24 h/day except when the chamber was opened briefly once or twice a day to remove rats or clean cages and replenish food and water. All rats were exposed to a 12:12-h light-dark cycle and were allowed free access to standard rat food (Prolab R-M-H 3000, Agway) and water.

Right ventricular hypertrophy. To assess the severity of pulmonary hypertension in the chronically hypoxic rats, the hearts were dissected, and an index of right ventricular hypertrophy was calculated as the ratio of the wet weight of the free wall of the right ventricle to the wet weight of the left ventricular wall plus septum.

Isolated perfused lungs. Three different groups of lungs were studied: normotensive lungs isolated from normoxic rats and ventilated with 21% O_{2}-5% CO_{2}-74% N_{2} (normoxic normotensive) and hypertensive lungs isolated from chronically hypoxic rats and ventilated with either 21% O_{2}-5% CO_{2}-74% N_{2} (normoxic hypertensive) or 0% O_{2}-5% CO_{2}-95% N_{2} (hypoxic hypertensive). Whereas chronically hypoxic rats for the study of normoxic hypertensive lungs were exposed to normoxia (21% O_{2} at P_{b} = 630 mmHg) for 30–45 min between removal from the hypobaric chamber and lung isolation, those for the study of hypoxic hypertensive lungs were exposed to normoxia (21% O_{2} at P_{b} = 630 mmHg) for 30–45 min between removal from the hypobaric chamber and lung isolation, those for the study of hypoxic hypertensive lungs were exposed to normoxia (21% O_{2}). The perfusate was a physiological salt solution (PSS) containing (in mM) 116.3 NaCl, 5.4 KCl, 0.83 MgSO_{4}, 19.0 NaHCO_{3}, 1.04 NaH_{2}PO_{4}, 1.8 CaCl_{2}, 2H_{2}O and 5.5 d-glucose (Earle’s balanced salt solution; Sigma). Ficoll (4 g/100 ml, type 70; Sigma) was included as a colloid, and 3.1 mM sodium medofenamate (Sigma) was added to inhibit synthesis of vasodilator prostaglandins (15, 26). After the lungs were flushed of blood with 20 ml of PSS, they were perfused with a recirculating volume of 50 ml. Effluent perfusate drained from a left ventricular cannula into a perfusate reservoir. Lung and perfusate temperatures were maintained at 38°C, and perfusate pH was kept between 7.3 and 7.4. The perfusate reservoir volume was monitored continuously, and any lung preparation that leaked perfusate or became edematous was excluded from the study. Mean perfusion pressure, which was monitored continuously with a transducer and pen recorder, was measured after 20 min of equilibration (initial pressure) and again at the end of the experiment after 65 min of perfusion (final pressure). The drugs used in the protocols described in Experimental protocols were added to the perfusate reservoir to achieve the desired circulating concentrations.

Experimental protocols. To determine whether the previously reported increase in perfusate level of NO_{x} in normoxia-ventilated, chronically hypoxic hypertensive rat lungs (17, 24) was influenced by duration of the hypoxic exposure, we first compared perfusate accumulation of NO_{x} over 65 min of recirculating perfusion in normoxia (21% O_{2})-ventilated normotensive and 1-, 2-, 4-, 8-, and 12-wk chronically hypoxic hypertensive lungs. Samples (1 ml) of perfusate were collected just before the lung was added to the circuit and again after 65 min of perfusion and stored at −20°C for up to 2 wk before NO_{x} levels were measured as described in Measurement of plasma, perfusate, and tissue NO_{x} and exhaled NO. Perfuse NO_{x} accumulation was calculated by subtracting the concentration of the first sample from that of the second sample. In some normotensive and 4-wk hypertensive lungs, perfusate NO_{x} concentration ([NO_{x}]) was also measured after 30 min of perfusion to determine whether the accumulation of NO_{x}, was linear. The results showed a slight increase in perfusate [NO_{x}] in 1-wk hypertensive lungs and equal marked increases in 2- to 12-wk hypertensive lungs. We next tested whether the increased basal accumulation in 1-, 2-, and 4-wk hypertensive lungs was reduced by hypoxic (0% O_{2}) ventilation. Additional experiments with 4-wk hypertensive lungs examined the effects of ventilation with a less severe degree of hypoxia (3% O_{2}) on basal NO_{x} accumulation and whether hypoxic (0% O_{2}) ventilation also reduced stimulation of NO_{x} production by the receptor-independent Ca^{2+} agonist thapsigargin (24). In the latter experiment, NO_{x} levels were measured in perfusate samples collected just before (at 30 min of perfusion) and 15 min after the addition of 0.1 μM thapsigargin (Sigma) to the perfusate of normoxia- and hypoxia-ventilated hypertensive lungs.

To characterize the degree of lung tissue hypoxia in the above and subsequent isolated lung experiments, percent O_{2} in the lung effluent and influent perfusates was measured with an in-line O_{2} electrode (M1-730 Oxygen Electrode, Microelectrodes) during normoxic (21% O_{2}) ventilation and after 30 min of hypoxic ventilation with either 0% or 3% O_{2}, and the respective O_{2} tensions were calculated ([ambient pressure (630 mmHg) − water vapor pressure (47 mmHg)]) × percent O_{2}/(100). During normoxic ventilation, effluent and influent O_{2} tensions were 118 ± 2 and 120 ± 1 mmHg, respectively (n = 6 lungs). Effluent and influent O_{2} tensions were, respectively, 3 ± 1 and 33 ± 4 mmHg during 0% O_{2} ventilation and 20 ± 1 and 46 ± 6 mmHg during 3% O_{2} ventilation (n = 3 lungs/group). The higher O_{2} tensions in influent vs. effluent perfusate during hypoxic ventilation reflected the fact that the perfusate reservoir was not sealed
and allowed some reoxygenation by room air during the recirculating perfusion.

Because the above experiments showed that hypoxic ventilation reduced perfusate NO\textsubscript{x} accumulation in hypertensive rat lungs and because Archer et al. (2) found that hypoxia altered the decomposition and partitioning of NO rather than its synthesis by iNOS in cultured mesangial cells, we next measured exhaled NO\textsubscript{x} in lungs tissue NO\textsubscript{x} to test whether hypoxia increased the partitioning of NO to either of these compartments. For the measurement of exhaled NO\textsubscript{x}, the total volume of gas expired between 30 and 60 min of perfusion of normoxic (21% O\textsubscript{2})-ventilated normotensive and 4-wk hypertensive lungs and hypoxia (0% O\textsubscript{2})-ventilated hypertensive lungs was collected in a Mylar balloon. The [NO\textsubscript{x}] in the expired gas was then immediately measured as described in Measurement of plasma, perfusate, and tissue NO\textsubscript{x} and exhaled NO\textsubscript{x}. In all lungs, the inspired gas mixtures were passed through a charcoal filter to scrub contaminating NO. For the measurement of tissue NO\textsubscript{x}, separate normoxia-ventilated normotensive and hypertensive lungs and hypoxia-ventilated hypertensive lungs were perfused for 65 min, flushed with ultrapure water (NANOpure, Barnstead), cleaned of extralobar vessels and airways, and homogenized in 50 ml of ultrapure water. The homogenates were centrifuged, and the supernatants were stored at −20°C for up to 2 wk before NO\textsubscript{x} levels were measured as described in Measurement of plasma, perfusate, and tissue NO\textsubscript{x} and exhaled NO\textsubscript{x}. To address whether the effect of hypoxic ventilation was related to a decrease in superoxide anion-mediated destruction of extracellular NO (2, 6), the final experiment in this series tested whether perfusion of normoxic hypertensive lungs with the scavenger of extracellular superoxide anion, 150 U/ml of superoxide dismutase (from bovine erythrocytes; Sigma) (6), would mimic the effect of hypoxic ventilation by inhibiting perfusate accumulation of NO\textsubscript{x}.

The preceding experiments indicated that hypoxic ventilation attenuated the increased NO\textsubscript{x} synthesis in isolated hypertensive rat lungs, and we next investigated whether hypoxia had a similar effect in vivo. Plasma [NO\textsubscript{x}] was measured as an index of NO production (50) in three groups of rats: normoxic rats breathing 21% O\textsubscript{2} at PB\textsubscript{50} = 630 mmHg, and 4-wk chronically hypoxic rats breathing 10% O\textsubscript{2} at PB\textsubscript{50} = 260 mmHg, and 4-wk chronically hypoxic rats allowed to breathe 21% O\textsubscript{2} at PB\textsubscript{50} = 630 mmHg for 1 h before the collection of blood samples. After the rats were anesthetized with pentobarbital sodium (30 mg ip) and ventilated with their respective inspired O\textsubscript{2} concentrations, i.e., 21 and 10% O\textsubscript{2}, the chest was opened, and heparinized blood samples (5 ml) were collected from the left ventricle. The blood was centrifuged, and the plasma samples were stored at −20°C for up to 2 wk before NO\textsubscript{x} levels were measured as described in Measurement of plasma, perfusate, and tissue NO\textsubscript{x} and exhaled NO\textsubscript{x}. Arterial and mixed venous blood O\textsubscript{2} tensions, which were measured in a separate group of pentobarbital sodium-anesthetized, catheterized, chronically hypoxic rats, were, respectively, 85 ± 3 and 42 ± 2 mmHg with the animals breathing 21% O\textsubscript{2} and 36 ± 4 and 26 ± 1 mmHg after 30 min of 10% O\textsubscript{2} (n = 4).

Our next series of experiments tested whether the hypoxic inhibition of perfusate NO\textsubscript{x} accumulation in 4-wk hypertensive lungs could be attributed to limitations in either the cosubstrate L-arginine (51) or the cofactor tetrahydrobiopterin (6) or to a decrease in influx of extracellular Ca\textsuperscript{2+} (41). Effects of L-arginine supplementation were examined by adding three different levels of L-arginine (10, 100, and 1,000 µM; Sigma) to the perfusate at the beginning of lung perfusion and measuring perfusate NO\textsubscript{x} after 65 min of hypoxic (0% O\textsubscript{2}) ventilation. The effect of a supplementary cofactor was similarly tested by adding 100 µM (6R)-5,6,7,8-tetrahydrobiopterin (Alexis) to the perfusate. This concentration of tetrahydrobiopterin has been found to restore impaired NO synthesis in isolated, prehypertensive rat aortas (6). We then tested whether perfusion of normoxic hypertensive lungs with Ca\textsuperscript{2+}-free PSS (1.8 mM CaCl\textsubscript{2} replaced by equimolar NaCl) containing 100 µM ET\textsubscript{A} blocker to inhibit Ca\textsuperscript{2+} influx would mimic the inhibitory effect of hypoxic ventilation on perfusate NO\textsubscript{x} accumulation. After the 65-min perfusion sample was collected for the measurement of basal NO\textsubscript{x} accumulation, 0.1 µM thapsigargin was added, and a second sample was collected 15 min later to determine whether the Ca\textsuperscript{2+}-free plus ET\textsubscript{A} blocker inhibited thapsigargin-induced stimulation of NO\textsubscript{x} production. The control normoxia-ventilated hypertensive lungs for this experiment were perfused with standard PSS.

Because the preceding experiment indicated that the increased basal accumulation of perfusate NO\textsubscript{x} in normoxic hypertensive lungs was not mediated by Ca\textsuperscript{2+} influx, we next investigated whether NO synthesis in these lungs was due to a constitutive Ca\textsuperscript{2+}-independent NOS or to the Ca\textsuperscript{2+}-independent iNOS. Hypertensive lungs were exposed from the beginning of perfusion to either the nonspecific NOS inhibitor N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 100 µM; Calbiochem) (10), the preferential iNOS inhibitor L-N\textsuperscript{G}-(1-iminoethyl)lysine (10 µM; Alexis) (10, 29), or the preferential iNOS and endothelial NOS inhibitors L-NAME (100 µM; Alexis) and perfusate [NO\textsubscript{x}] was measured 65 min later. The concentrations of the NOS inhibitors were chosen on the basis of reports (10, 29, 45) of their relative selectivity for the three NOS isoforms. NO\textsubscript{x} accumulation was also measured in another group of 4-wk hypertensive lungs perfused with Ca\textsuperscript{2+}-free PSS containing the chelator of intracellular Ca\textsuperscript{2+}, 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM; 5 µM; Sigma) (16).

To test whether the increased basal accumulation of perfusate NO\textsubscript{x} in normoxia-ventilated 4-wk hypertensive lungs was due to activation of ET\textsubscript{A} receptors by endogenous ET-1 (16, 22, 23), the selective ET\textsubscript{A} and ET\textsubscript{B} receptor blockers BQ-788 and BQ-123, respectively (1 µM; Banyu Pharmaceutical), were added to the perfusate of separate lungs at the beginning of perfusion, and NO\textsubscript{x} accumulation was measured over 65 min. A previous study (36) of PSS-perfused rat lungs showed that 1 µM concentrations of BQ-788 and BQ-123 selectively inhibited ET\textsubscript{B} and ET\textsubscript{A} receptors, respectively. Dimethyl sulfoxide (DMSO; 0.01%), the solvent for BQ-788, was added to the perfusate of vehicle control hypertensive lungs. The results showed that the ET\textsubscript{B} receptor blocker inhibited basal NO\textsubscript{x} accumulation, and to test its effects on stimulated NO\textsubscript{x} production, additional normoxia-ventilated hypertensive lungs were pretreated for 30 min with either vehicle or 1 µM BQ-788 before the addition of 0.1 µM thapsigargin and measurement of the accumulation of perfusate NO\textsubscript{x} over 15 min. Our earlier in vivo experiment showed that normoxic ventilation increased plasma levels of NO\textsubscript{x} in chronically hypoxic rats, and to test whether this response was also inhibited by ET\textsubscript{B} receptor blockade, we pretreated pentobarbital sodium-anesthetized rats ventilated with 10% O\textsubscript{2} with either the vehicle DMSO (1 µl kg\textsuperscript{−1} min\textsuperscript{−1} iv) or BQ-788 (10 nmol kg\textsuperscript{−1} min\textsuperscript{−1} iv) for 30 min before ventilation was switched to 21% O\textsubscript{2} for the next 60 min. Infusion of DMSO or BQ-788 was continued throughout the procedure. Heparinized blood samples (0.2 ml) were collected via a carotid artery catheter just before and at 30 and 60 min of normoxic ventilation for the measurement of plasma NO\textsubscript{x} as described in Measurement of plasma, perfusate, and tissue NO\textsubscript{x} and exhaled NO\textsubscript{x}. The dose of BQ-788 used in this experiment has been previously shown to block ET\textsubscript{B} receptors in intact rats (34).
Measurement of plasma, perfusate, and tissue NOx and exhaled NO. An NO chemiluminescence analyzer (NOA 280, Sievers Research) was used to measure the levels of NOx (NO, NO2, NO3, nitrosothiols, and peroxynitrite) in plasma (50), lung perfusate (17, 19, 24, 25), and lung tissue (35) and of NO in expired air (5, 7, 13, 25). Aliquots of plasma (1 µl), perfusate (10 µl), and tissue supernatant (10 µl) samples were added to 2 ml of 0.1 M vanadium chloride (type III; Aldrich) dissolved in 1 N HCl and heated to 90°C in the purge vessel of the NO analyzer to reduce all NOx to NO. The liberated NO was driven into the chemiluminescence chamber by bubbling the reaction mixture with argon. Calibration curves for NOx levels were generated daily by measuring the amount of NO produced by a range (10–100 µM) of sodium nitrate solutions (Mallinckrodt). The concentration of NO in expired air samples was measured by drawing gas directly from the Mylar balloon through a hygroscopic perfluorinated ion-exchange membrane (Nafion) drier into the chemiluminescence chamber of the analyzer. Calibration curves for NO concentration were generated daily with air containing <1 part/billion (ppb) NO and 45 parts/million NO calibration gas (Ohmeda). The lower limit of NO detection was 0.1 ppb.

Statistics. Data are expressed as means ± SE. Statistical analysis was done by Student's t-test or analysis of variance (ANOVA) with the Fisher post hoc test for multiple comparisons. Differences were considered significant at \( P < 0.05 \).

RESULTS

Right ventricular hypertrophy. The severity of pulmonary hypertension in 1-, 2-, 4-, 8-, and 12-wk chronically hypoxic rats was reflected in the increased ratios of right ventricular to left ventricular plus septal weights that were 43 ± 3 (n = 8), 56 ± 4 (n = 8), 59 ± 2 (n = 26), 62 ± 6 (n = 3), and 66 ± 6% (n = 6), respectively, vs. 26 ± 2% (n = 8) in normoxic normotensive control rats.

Hypoxia inhibits increased perfusate NOx in chronically hypoxic hypertensive rat lungs. Figure 1 confirms and extends to a range of duration of exposure to chronic hypoxia previous observations by Muramatsu et al. (24) and Isaacson et al. (17) that hypertensive lungs isolated from chronically hypoxic rats and ventilated with 21% O2 accumulate increased levels of NOx compared with control hypertensive rat lungs.

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In contrast to normoxic ventilation, when chronically hypoxic hypertensive lungs were kept hypoxic by ventilation with 0% O2, perfusate accumulation of NOx was markedly reduced and was not significantly greater than that in normoxic normotensive lungs (Fig. 3). The threshold for hypoxic inhibition of NOx accumulation in this particular isolated lung preparation was apparently near an inspired O2 concentration of 3% because perfusate [NOx] after 65 min of perfusion in 4-wk hypertensive lungs ventilated with 3% O2 tended to be less than that of hypertensive lungs ventilated with 21% O2 (0.53 ± 0.09 vs. 0.80 ± 0.10 µM; n = 4 and 13, respectively; \( P = 0.1 \)). In addition to suppressing basal NOx accumulation in 4-wk hypertensive lungs, hypoxic (0% O2) ventilation also inhibited thapsigargin-induced stimulation of NOx accumulation (increase in perfusate [NOx] 15 min after the addition of 0.1 µM thapsigargin was 0.23 ± 0.03 µM in four normoxic lungs vs. 0.09 ± 0.03 µM in four hypoxic lungs; \( P < 0.05 \)).

To examine whether hypoxic inhibition of perfusate NOx accumulation was due to differences in partitioning of NO (2) instead of to decreased synthesis, the effects of hypoxic ventilation on levels of exhaled NO and tissue NOx were measured in 4-wk normotensive and normoxic hypertensive lungs. The average [NO] in expired gas collected between 30 and 60 min of perfusion was not different between normoxic normotensive and normoxic hypertensive lungs (5.7 ± 1.5 and 5.7 ± 0.2 ppb, respectively) and was not altered during chronic hypoxic ventilation (0% O2) in any group of lungs.
of hypertensive lungs (6.3 ± 0.6 ppb; n = 3/group).
Similarly, tissue [NOx] measured after 65 min of perfusion was not different among normoxic normotensive, normoxic hypertensive, and hypoxic hypertensive lungs (0.43 ± 0.12, 0.39 ± 0.09, and 0.41 ± 0.06 pM/µg protein, respectively; n = 4/group). In addition, perfusion of normoxia-ventilated hypertensive lungs with the scavenger of extracellular superoxide anion superoxide dismutase did not reduce perfusate accumulation of NOx (perfusate [NO] after 65 min of perfusion was 1.2 ± 0.5 µM; n = 3).

Hypoxia reduces plasma NO levels in chronically hypoxic rats. To test whether hypoxic inhibition of NO production in chronically hypoxic hypertensive lungs also occurred in vivo, we compared the effects of normoxic (21% O2) and hypoxic (10% O2) ventilation on plasma [NOx] in 4-wk chronically hypoxic rats. Whereas there was no difference in plasma NOx levels between normoxic rats breathing 21% O2 (11.2 ± 0.6 µM; n = 9) and chronically hypoxic rats breathing 10% O2 (11.9 ± 0.7 µM; n = 23), the levels were increased (16.9 ± 0.7 µM; P < 0.05; n = 11) in chronically hypoxic rats breathing 21% O2 for 1 h before blood samples were collected.

L-Arginine and tetrahydrobiopterin do not prevent and Ca2+-free perfusion does not mimic hypoxic inhibition of NOx accumulation in chronically hypoxic hypertensive rat lungs. The results in Hypoxia inhibits increased perfusate NOx in chronically hypoxic hypertensive rat lungs suggested that hypoxia inhibited the increased NO synthesis in hypertensive rat lungs, and we next evaluated whether this inhibition could be attributed to limitations in either L-arginine, tetrahydrobiopterin, or Ca2+ influx. Figure 4 shows that the addition of either L-arginine or tetrahydrobiopterin to the perfusate of 4-wk hypertensive lungs did not prevent the hypoxic inhibition of perfusate NOx accumulation. Perfusate of normoxia-ventilated hypertensive lungs with Ca2+-free PSS containing 100 µM EGTA did not reduce the increased basal accumulation of perfusate NOx but did inhibit the thapsigargin-induced stimulation of NO production (Fig. 5).

Inhibitors of constitutive NOS, but not of iNOS, reduce increased perfusate NOx in normoxic hypertensive rat lungs. To investigate whether the increased perfusate NOx in normoxic hypertensive lungs was due to a constitutive, Ca2+-dependent NOS or to the Ca2+-independent iNOS, effects of the NOS inhibitors L-NMMA, L-N6-(1-iminoethyl)lysine, and S-isopropylisothiourea and the chelator of intracellular Ca2+ BAPTA-AM were examined in normoxia-ventilated 4-wk hypertensive lungs. The increased accumulation of NOx was significantly reduced by L-NMMA but not by either L-N6-(1-iminoethyl)lysine or S-isopropylisothiourea (Fig. 6).

ETB receptor but not ETA receptor blocker inhibits increased perfusate NOx in chronically hypoxic hypertensive rat lungs. In view of evidence that ET-1 stimulates endothelial NO synthesis via the ETB receptor (16) and that
endogenous ET-1 vasoactivity is increased in hypertensive rat lungs (22, 23), we tested whether the high normoxic level of NOx production could be attributed to ETB-receptor activity. Figure 7 shows that although the specific ETB-receptor antagonist BQ-788 completely prevented the increased basal accumulation of perfusate NOx in normoxic hypertensive lungs, the ETA-receptor antagonist BQ-123 had no effect. In contrast to hypoxic ventilation that reduced both basal and thapsigargin-stimulated NOx accumulation (see L-Arginine and tetrahydrobiopterin do not prevent and Ca\textsuperscript{2+}-free perfusion does not mimic hypoxic inhibition of NOx accumulation in chronically hypoxic hypertensive lungs), BQ-788 did not inhibit the response to the receptor-independent Ca\textsuperscript{2+} agonist (increase in perfusate [NOx] 15 min after the addition of 0.1 µM thapsigargin was 0.23 ± 0.05 µM in three vehicle control hypertensive lungs and 0.20 ± 0.10 µM in three hypertensive lungs pretreated for 30 min with 1 µM BQ-788). Similar to the above finding in isolated hypertensive lungs, BQ-788 also inhibited the normoxia-induced increase in plasma NOx levels in intact chronically hypoxic rats. During 30 and 60 min of ventilation with 21% O\textsubscript{2}, plasma [NOx] increased from 8.0 ± 0.4 to 11.0 ± 0.5 and 10.7 ± 0.4 µM, respectively, in three vehicle control rats (P < 0.05) but did not change in three BQ-788-treated rats (8.8 ± 0.9 vs. 8.8 ± 0.1 and 8.9 ± 0.4 µM, respectively). It is unclear why the levels of plasma NOx in this experiment were lower than those in the earlier in vivo experiment (see Hypoxia reduces plasma NOx levels in chronically hypoxic rats). Possible explanations include the effects of the longer period of anesthesia or the infusion of DMSO in this experiment.

Fig. 5. Perfusion with Ca\textsuperscript{2+}-free physiological salt solution (PSS) containing 100 µM EGTA does not reduce basal accumulation of perfusate NOx but does inhibit thapsigargin (TG)-stimulated accumulation in normoxia (21% O\textsubscript{2})-ventilated 4-wk chronically hypoxic HL. Basal accumulation of NOx was measured after 65 min of recirculating perfusion, and stimulated accumulation was measured 15 min after addition of 0.1 µM TG to perfusate. Control normoxic hypertensive lungs were perfused with standard PSS. Values are means ± SE; nos. in parentheses, no. of lungs. *P < 0.05 vs. respective control by t-test.

Fig. 6. Perfusate accumulation of NOx in normoxia (21% O\textsubscript{2})-ventilated chronically hypoxic HL is inhibited by nonselective NO synthase (NOS) inhibitor N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA; 100 µM) and intracellular Ca\textsuperscript{2+} chelator 1,2-bis(2-aminophenoxy)ethane-N,N,N\textsuperscript{8},N\textsuperscript{8}-tetraacetic acid (BAPTA) tetrakis-AM (5 µM) but not by either selective inducible NOS inhibitor L-N\textsuperscript{6}-(1-iminoethyl)lysine (L-NIL; 10 µM) or preferential inhibitor of inducible NOS and neuronal NOS S-isopropylisothiourea (ITU; 10 µM). Each inhibitor was added to perfusate at beginning of experiment, and perfusate NOx was measured after 65 min of recirculating perfusion. CON, control group. Values are means ± SE; nos. in parentheses, no. of lungs. *P < 0.05 vs. CON by ANOVA.

Fig. 7. Perfusate accumulation of NOx in normoxia (21% O\textsubscript{2})-ventilated chronically hypoxic HL is prevented by endothelin type B-receptor blocker BQ-788 (1 µM) but not by endothelin type A-receptor blocker BQ-123 (1 µM). BQ-788, BQ-123, or 0.01% DMSO (vehicle control) was added to perfusate at beginning of experiment, and NOx was measured after 65 min of recirculating perfusion. Values are means ± SE; n = 4 lungs/group. *P < 0.05 vs. control by ANOVA.
Initial pressure was measured at 20 min of perfusion. Final pressure
in chronically hypoxic hypertensive rat lungs. HL, hypertensive lungs
from rats exposed to chronic hypoxia. Normotensive and hypertensive
rat lungs Table 1. Perfusion pressures in normoxia-ventilated
normotensive and hypertensive rat lungs

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<th>Perfusion Pressure, mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
</tr>
<tr>
<td>NL</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>HL</td>
<td>7.1 ± 0.1</td>
</tr>
<tr>
<td>1 wk</td>
<td>10.3 ± 0.6*</td>
</tr>
<tr>
<td>2 wk</td>
<td>10.2 ± 0.7*</td>
</tr>
<tr>
<td>4 wk</td>
<td>8.4 ± 0.7*</td>
</tr>
<tr>
<td>8 wk</td>
<td>9.4 ± 0.3*</td>
</tr>
<tr>
<td>12 wk</td>
<td>6.0 ± 0.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of lungs. NL, normotensive control lungs; HL, hypertensive lungs from rats exposed to chronic hypoxia. Initial pressure was measured at 20 min of perfusion. Final pressure was measured at 65 min of perfusion. *Significant difference from respective NL value, P < 0.05 by ANOVA.

Effects of inhibitors of NOx accumulation on perfusion pressure in chronically hypoxic hypertensive rat lungs. Initial (20-min) and final (65-min) perfusion pressures in the normoxia-ventilated normotensive and hypertensive lungs of Fig. 1 are shown in Table 1. The pressures were higher in 2-, 4-, 8-, and 12-wk hypertensive lungs than in normotensive lungs, and the increases in pressure during 45 min of normoxic perfusion were greater in 1- and 2-, and 4-wk hypertensive lungs (data not shown). The increases in pressure in 45 min of hypoxic perfusion of 1-, 2-, and 4-wk hypertensive lungs were 4.1 ± 0.9, 3.5 ± 0.9, and 2.9 ± 0.6 mmHg, respectively, which were not different from the increases in the respective normoxia-ventilated hypertensive lungs (see Table 1). There were no differences in initial pressures among the five groups of hypertensive lungs displayed in Fig. 6, but increases in pressure during the following 45 min of perfusion were greater in L-NMMA-treated lungs than in control and other inhibitor-treated groups (Table 2). In the experiment with ET-1-receptor antagonists (Fig. 7), there were no differences in initial pressure among the three groups of lungs (data not shown), and although the subsequent increases in pressure tended to be greater in BQ-788-treated lungs than in vehicle control (5.7 ± 0.5 mmHg) and BQ-123 (6.4 ± 0.9 mmHg) lungs, the differences were not significant.

DISCUSSION

The major findings of this study were that the increased basal accumulation of perfusate NOx in normoxia-ventilated, chronically hypoxic hypertensive rat lungs was markedly inhibited by hypoxic ventilation and completely prevented by an ETB-receptor blocker. Similarly, ETB-receptor blockade also inhibited a normoxia-induced increase in plasma NOx in intact chronically hypoxic rats. These results suggest that low O2 tension suppresses an endogenous ET-1-induced, ETB receptor-mediated stimulation of NO synthesis in hypoxia-induced hypertensive rat lungs.

Our initial experiment confirmed previous reports (17, 24) of increased perfusate accumulation of NOx in normoxia-ventilated hypertensive rat lungs and showed that the increased production of NOx began to appear within 1 wk of hypoxic exposure, reached a maximum by 2–4 wk, and persisted for as long as 12 wk. This time course of increased normoxic NOx production is similar to that of increased gene expression for eNOS (21, 28, 37, 47, 48), iNOS (21, 47, 48), and nNOS (37, 47) in the hypertensive lungs of chronically hypoxic rats. It is likely that increased peripheral pulmonary arterial expression of eNOS protein (21, 28, 47) accounts for most of the enhanced responsiveness to endothelium-dependent vasodilators that has been found in hypertensive rat lungs (4, 17, 24, 26, 30, 33), but the source of the increased level of perfusate NOx is uncertain.

Our results suggested that the high normoxic NOx production was due largely to activity of a Ca2+-dependent isoform of NOS, i.e., eNOS and/or nNOS, because it was inhibited by the chelator of intracellular Ca2+ BAPTA-AM (16) and the nonselective NOS inhibitor L-NMMA (10, 29) but not by the preferential iNOS blocker L-N6-(1-iminoethyl)lysine (10). The lack of effect of S-isopropylisothiourea, an inhibitor with reported selectivity for iNOS and nNOS over eNOS (45), also supported involvement of eNOS in the NOx production. Although it could be questioned whether a constitutive NOS was involved because inhibition of Ca2+-free plus EGTA perfusion did not reduce NOx accumulation, some agonists such as Ca2+-free plus EGTA perfusion did not reduce NOx accumulation, some agonists such as ET-1 can stimulate eNOS by inducing release of intracellular Ca2+ in the absence of Ca2+-influx (16). Thus our observation that increased basal NOx production was not inhibited by extracellular EGTA but was markedly reduced by intracellular BAPTA-AM could be explained by an underlying ET-1-induced stimulation of NO synthesis that was not mediated by Ca2+-influx.

In contrast to the elevated levels of eNOS protein and normoxic NOx production in hypertensive lungs of chronically hypoxic rats, hypertensive lungs of chronically hypoxic newborn pigs have decreased levels of both eNOS protein and normoxic NOx production (11). Similarly, Tyler et al. (42) found in another study that hypertensive lungs from both monocrotaline-treated and fawn-hooded rats express low levels of eNOS protein and produce low levels of perfusate NOx. Thus,
in hypertensive lungs, there appears to be a correlation between upregulation of eNOS and increased normoxic production of NOx. However, because eNOS is found in both endothelial and epithelial cells in rat lungs (46) and because nNOS may also be upregulated in chronically hypoxic rat lungs (37, 47), it is uncertain whether pulmonary arterial eNOS is the primary source of the increased perfusate NOx. Contributing to this uncertainty is that although whole lung levels of eNOS protein are low in monocrotaline-treated and fawn-hooded hypertensive lungs, immunohistochemical staining suggests that the hypertensive pulmonary arterial levels are similar to those in chronically hypoxic rats (28, 42). If so and if eNOS is the primary source of perfusate NOx, then why is normoxic NOx production increased only in the hypoxia-induced hypertensive lungs?

A possible answer relates to the almost complete inhibition of normoxic NOx accumulation in hypoxia-induced hypertensive lungs by the ETB-receptor blocker BQ-788. In conjunction with evidence for increased ET-1 and ET-A- and ETB-receptor gene expression (22) and increased endogenous ET-1 vasoactivity in chronically hypoxic hypertensive rat lungs (23), this finding suggested that the high NOx production was driven by inherent stimulation of endothelial cell ETB receptors. This idea is further supported by a preliminary report by Soma et al. (40) of marked increases in the number of endothelial ETB receptors in the peripheral pulmonary arteries of chronically hypoxic rats. Whether a similar upregulation of pulmonary arterial endothelial ETB receptors occurs in monocrotaline-treated and fawn-hooded hypertensive lungs remains to be determined. However, if it does not, and one study has found decreased levels of ETB-receptor mRNA in homogenates of monocrotaline-treated lungs (49), then the difference in normoxic NOx production between hypoxia-induced hypertensive lungs and monocrotaline-treated and fawn-hooded hypertensive lungs (42) might be due to differences in ETB receptor-mediated stimulation of eNOS.

Numerous studies of NOS enzyme preparations (1, 8, 27), cultured endothelial cells (3, 44), pulmonary artery rings (18, 32, 38), perfused normotensive lungs (5, 7, 13, 19, 25), and normal human subjects (8) have found evidence for acute hypoxic inhibition of NO synthesis. Our study extends these observations by indicating that hypoxia also inhibits NO production in chronically hypoxic hypertensive rat lungs that express elevated levels of NOS (21, 28, 37, 47, 48). Similar to studies in neonatal pig lungs (25) and normal rabbit lungs (19), we found in hypertensive rat lungs that hypoxic ventilation reduced the accumulation of perfusate NOx. However, in contrast to the decreases observed in isolated pig (7, 25) and rabbit (5, 13) lungs and in normal human subjects (8), we measured no decrease in the already low levels of exhaled NO during hypoxic ventilation of hypertensive rat lungs. We do not know whether the absence of a decrease in our study was due to the difference in species, normal vs. chronically hypoxic hypertensive lungs, or another unidentified factor. Also, except to interpret the high level of perfusate NOx as being due to a localized intravascular production, we have no definitive explanation of why exhaled NO was not increased in the normoxia-ventilated hypertensive lungs. If the increased NO was synthesized primarily by the increased levels of eNOS in the hypertensive pulmonary arteries (21, 28) and converted locally to NOx, it would then not necessarily show up in exhaled air.

The level of NOx in the incubation medium of cultured cells (44), the perfusate of isolated lungs (13, 17, 19, 24, 25), and the plasma of intact animals (50) is generally believed to reflect the rate of NO synthesis. However, Archer et al. (2) have emphasized that decreases in O2 tension also slow decomposition of NO to nitrite, nitrate, and other reaction products and have shown in cultured mesangial cells that hypoxia reduces medium [NOx] independently of the rate of NO synthesis by allowing NO to partition to the headspace of the incubation flask. Our measurements of the effects of hypoxic ventilation on exhaled NO and tissue NOx in hypertensive lungs provided no evidence that hypoxic inhibition of perfusate NOx accumulation was due to redistribution of NO away from perfusate. Furthermore, our finding that perfusion of normoxic hypertensive lungs with superoxide dismutase did not mimic the effects of hypoxic ventilation indicated that hypoxic inhibition was not due to a decrease in superoxide anion-mediated destruction of extracellular NO. Thus, unless NO was partitioned into a cellular component that was not detected by measurement of tissue NOx, it is unlikely that hypoxic inhibition of perfusate NOx accumulation was due to a decrease in NO decomposi- tion and redistribution away from the perfusate instead of to a decrease in NO synthesis.

Our measurements of plasma NOx in intact rats were consistent with the results of the isolated lung experiments. Thus, although chronically hypoxic rats had normal levels of plasma NOx during hypoxic (10% O2) ventilation, there was a significant increase in [NOx] after 1 h of normoxic (21% O2) ventilation. Unless normoxia caused a decrease in either the extracellular volume of distribution of NOx (including the relatively small plasma volume) or the rate of renal excretion of nitrate, the ~40% increase in plasma [NOx] likely reflected an even larger increase in NO synthesis (50). Another similarity of intact rats to isolated lungs was that the normoxia-induced increase in plasma NOx was inhibited by BQ-788. These results provided no direct evidence that the lung was the primary source of the increase in plasma NOx, but did indicate that hypoxic ventilation suppressed a similar increased capacity for ETB receptor-mediated NO synthesis in both intact chronically hypoxic rats and isolated hypertensive lungs.

Because of the difference in inspired O2 used in hypoxic isolated lungs (0% O2) vs. hypoxic intact rats (10% O2), the question arises as to whether the in vitro results are relevant to the in vivo conditions. Although measurements of O2 tension in both effluent and influent perfusates of isolated lungs and arterial and mixed
venous blood of intact rats confirmed the expected difference in severity of airway hypoxia, \(O_2\) tension in the influent perfusate of hypoxic lungs (\(30\) mmHg) was similar to that in mixed venous blood of hypoxic rats (\(26\) mmHg). Depending on which cells were responsible for increased perfusate NO in normoxic hypertensive lungs, these results were consistent with the idea that hypoxic inhibition of NO synthesis occurred at physiologically relevant \(O_2\) tensions. For example, if increased NO production was due primarily to the increased eNOS in the endothelium of hypertensive pulmonary arteries (21, 28), then hypoxic inhibition was likely occurring at an \(O_2\) tension closer to that of the influent perfusate (\(30\) mmHg) than that of airway gas (\(3\) mmHg). Thus, because of the design of the isolated lung setup that allowed some reoxygenation of perfusate and possibly also because of undefined decreases in the sensitivity of “\(O_2\) sensing” in the in vitro preparations, it takes a more severe degree of airway hypoxia in PSS-perfused rat lungs to mimic the effects of airway hypoxia in intact rats.

Although hypoxic inhibition of NO synthesis in perfused lungs and intact animals could theoretically involve interference with the supply of the cosubstrates \(L\)-arginine and NADPH, the cofactor tetrahydrobiopterin, or \(Ca^{2+}\) (in the case of eNOS and nNOS), in vitro studies (1, 27) of all three NOS isoforms show that a reduction in the supply of the cosubstrate \(O_2\) inhibits enzyme activity in the presence of saturating concentrations of the other cosubstrates and cofactors. The estimated Michaelis-Menten constant values for \(O_2\) have varied among studies and NOS isoforms, ranging from as low as \(-6\) \(\mu\)M for iNOS (27) to as high as \(\sim 400\) \(\mu\)M for nNOS (1), but it is apparent that the rate of NO synthesis by all three isoforms can be directly regulated by physiological levels of \(O_2\). Our finding of hypoxic inhibition of basal accumulation of perfusate NO in hypertensive rat lungs is consistent with this concept. We found no evidence that hypoxic inhibition was due to a limitation of either \(L\)-arginine or tetrahydrobiopterin, and it could not be attributed to decreased \(Ca^{2+}\) influx. The possible role of a decrease in cosubstrate NADPH was not evaluated in our study. However, because enzymes of the hexose monophosphate shunt are upregulated and NADPH levels are preserved in the lungs of rats exposed to hypoxia for 5 days (43), it is unlikely that hypoxic ventilation reduces the level of this electron donor in hypoxia-induced hypertensive rat lungs.

Although not a universal finding (30), numerous studies (4, 17, 24, 26, 33) have shown that inhibitors of NO synthesis cause a marked, sustained vasoconstriction in hypertensive rat lungs. In contrast, the present experiments showed that although inhibition of NO\(_x\) accumulation by \(L\)-NMMA was associated with an increase in vascular tone, inhibition by hypoxia or BAPTA-AM was not. The absence of vasoconstriction with BAPTA-AM was likely due to a direct inhibition of vascular smooth muscle contractility. Because the response to sustained hypoxia is biphasic in PSS-perfused rat lungs, i.e., vasoconstriction followed by vasodilation (15), a similar effect might have accounted for the absence of increased vascular tone during the 65 min of hypoxic ventilation. There was a tendency for the inhibition of NO\(_x\) accumulation with BQ-788 to be accompanied by an increase in perfusion pressure, but the increase was not markedly different from that in either the vehicle control or BQ-123-treated lungs. Thus these findings indicated that there was not a simple inverse relationship between inhibition of accumulation of perfusate NO\(_x\) and an increase in vascular tone in the PSS-perfused hypertensive lungs. It is unclear whether the more vigorous vasoconstrictr responses previously observed in hypertensive lungs with the \(L\)-arginine analogs nitro-L-arginine and nitro-L-arginine methyl ester (4, 17, 24, 26, 33) were due to more effective inhibition of NO synthesis or to nonspecific effects of the inhibitors (17). We did not use these NOS inhibitors in this study because of their interference with the NO\(_x\) assay (12).

In summary, this study showed that an increased capacity for NO\(_x\) production in both hypoxia-induced hypertensive rat lungs and intact chronically hypoxic lungs was inhibited by either hypoxic ventilation or ET\(_B\)-receptor blockade. The results suggested that limitation of the supply of the essential cosubstrate molecular \(O_2\) to NOS counteracted an endogenous ET-1-induced, ET\(_B\)-mediated stimulation of NO synthesis. Thus, despite the increased levels of pulmonary arterial eNOS protein in chronically hypoxic rats, it is possible that hypoxic inhibition of NO production contributes to the development of pulmonary hypertension at least partly by reducing NO-mediated attenuation of ET-1-induced vasoconstriction (23, 26).

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REFERENCES


