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Hypoxia potentiates nitric oxide synthesis and transiently increases cytosolic calcium levels in pulmonary artery endothelial cells

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Hypoxia potentiates nitric oxide synthesis and transiently increases cytosolic calcium levels in pulmonary artery endothelial cells. V. Hampl, D.N. Cornfield, N.J. Cowan, S.L. Archer. ©ERS Journals Ltd 1995.

ABSTRACT: There is indirect, contradictory evidence both for increased and reduced synthesis of the endothelium-derived vasodilator, nitric oxide, in the pulmonary circulation during acute hypoxia. Therefore, we decided to directly measure the effect of acute hypoxia on nitric oxide production by cultured pulmonary endothelium. Because increases in the intracellular free calcium concentration are known to initiate nitric oxide synthesis, we also studied cytosolic calcium levels.

We measured the accumulation of the stable nitric oxide metabolite, nitrite, in the fluid used to superfuse the cultured bovine pulmonary artery endothelial cells at an oxygen tension (P_{O_2}) of either 20.3 (normoxia) or 4.9 kPa (hypoxia) (152 or 37 mmHg). Intracellular calcium levels were measured with dual-excitation microfluorimetry after loading the cells with the fluorescent calcium indicator, fura 2.

Basal NO synthesis, measured as nitrite accumulation over 10 min, was significantly higher under hypoxic than normoxic conditions (8.3±2.2 *versus* 4.6±0.8 nM). Hypoxia transiently increased cytosolic calcium concentration (from 113±10 to 231±45 nM). Ryanodine and thapsigargin (which deplete intracellular calcium stores), but not the removal of extracellular calcium, inhibited the hypoxic increase in cytosolic calcium, indicating that it resulted primarily from release of intracellular calcium. Bradykinin-elicited NO synthesis was potentiated by hypoxia. Bradykinin-induced increase in cytosolic calcium was not inhibited by hypoxia.

We conclude that hypoxia acutely increases cytosolic calcium levels and basal and bradykinin-stimulated nitric oxide synthesis in pulmonary artery endothelium.
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Moderate hypoxia is an important regulator of pulmonary vascular tone. Hypoxic pulmonary vasoconstriction (HPV) is a mechanism intrinsic to the pulmonary vascular smooth muscle [1]. The magnitude of HPV, however, is modulated by the endothelium. It has been reported that inhibitors of synthesis of the endothelium-derived vasodilator, nitric oxide (NO), potentiate HPV [2–14], indicating elevated pulmonary NO synthesis in hypoxia. In contrast, other studies have reported that hypoxia reduced pulmonary endothelium-dependent vasodilation and diminished accumulation of cyclic guanosine 3',5'-monophosphate (a second messenger of NO and other stimuli), suggesting that hypoxia reduced NO activity [15–19]. The aim of the present work was to address this discrepancy by directly measuring pulmonary endothelial NO production during hypoxia. The results demonstrate that the basal and bradykinin (Bk)-evoked production of NO is greater under hypoxic than normoxic conditions in cultured pulmonary artery endothelial cells.

Increases in endothelial cytosolic free calcium concentration, $[Ca^{2+}]_i$, precede, and are thought to cause, activation of NO synthase [20] and NO synthesis [21–23]. In this respect, both final steady-state levels and initial transient changes may be important. STEVENS *et al.* [24] have shown, in cultured pulmonary artery endothelial cells, that $[Ca^{2+}]_i$ levels decrease after ~10 min of hypoxia [24]. However, they have not reported rapid changes in $[Ca^{2+}]_i$.

We hypothesized that the increase in NO synthesis with hypoxia, observed in the first part of the current study, may be triggered by an initial transient increase in $[Ca^{2+}]_i$. To test this hypothesis, we measured $[Ca^{2+}]_i$ in cultured pulmonary artery endothelial cells during the first minutes of hypoxia. In addition, the source of the observed transient $[Ca^{2+}]_i$ increase was examined by removing calcium (Ca^{2+}) from the superfusate or by depleting intracellular Ca^{2+} stores with thapsigargin [25] or ryanodine [26] prior to the hypoxic challenge.

Methods

Cells

Bovine pulmonary artery endothelial cells (*Bos taurus* Line No. CCL209) were purchased from American Type

Culture Collection, Rockville, MD, USA. The cells between passage 16–25 were grown to confluence on customized culture plates using standard growth media, as described previously [27].

Study design

The study consisted of two separate experimental settings: measurement of NO production, and measurement of $[Ca^{2+}]i$. In both settings, the cells were superfused in the plates with normoxic or hypoxic solution. As NO is rapidly oxidized to nitrite (NO_2^-) in aqueous solutions [28], both NO and NO_2^- must be measured in order to determine NO production accurately. Therefore, NO production was measured as $NO+NO_2^-$ accumulation in the superfusate over 10 min periods of either hypoxia or normoxia. Nitrate (NO_3^-) was not measured, as its production from NO and NO_2^- in aqueous solutions is negligible in the absence of oxyhaemoproteins [28]. For the $[Ca^{2+}]i$ measurements the cells were loaded with the Ca^{2+} sensitive fluorophore, fura 2, and superfused on a microscope stage of a Ca^{2+} imaging spectrofluorimetric system. To test whether the observed hypoxic $[Ca^{2+}]_i$ spike is dependent on the entry of extracellular Ca^{2+}, 12 plates were perfused with nominally Ca^{2+} free solution. In addition, the role of intracellular Ca^{2+} stores was studied by depleting them with thapsigargin or ryanodine.

Superfusion of the cells

The plates were perfused on the microscope stage with Hank's balanced salt solution (HBSS; buffered to pH 7.4 with hydroxyethylpiperazine ethanesulphonic acid (HEPES)) at 14 ml·min^{-1}, in a recirculating manner (total recirculating volume was 40 ml). Calcium (1 mM) was added to both reservoirs after the start of perfusion. Partial pressure of oxygen (Po_2) at the cell surface was altered by switching between the normoxic (bubbled with 20 $\%O_2$, 5 $\%CO_2$, balance N_2) and hypoxic (bubbled with 0 $\%O_2$, 5 $\%CO_2$, balance N_2) reservoir. The pH of HBSS, reduced by CO_2 bubbling, was readjusted to 7.4 by sodium bicarbonate before the start of perfusion. Prior to exposure to hypoxic perfusate, the normoxic perfusate was aspirated from the plate to accelerate the change in Po_2. Similarly, hypoxic perfusate was removed from the plate before switching to normoxia. To improve the control of Po_2 at the cellular surface, the gas space below the lid was aerated with 100% N_2. The tip of a small (diameter=3 mm) oxygen electrode (MI-730 electrode connected to OM-4 Oxygen Meter, both from Microelectrodes Inc., Londonderry, NH, USA) was placed in the perfusate in the middle of the plate to monitor Po_2. Switching from normoxic to hypoxic perfusate dropped the O_2 saturation rapidly (~30–45 s) from 20.1% (Po_2 = 20.3 kPa (152 mmHg)) to 4.8% (Po_2 = 4.9 kPa (37 mmHg)). Pco_2 and pH did not change significantly with hypoxia.

NO measurement

$NO+NO_2^-$ accumulation in the superfusate of the cells was measured using an acidified version of an NO chemiluminescence assay [14]. Nitrite was first converted to NO by adding potassium iodide (a reducing agent, 0.2 ml of 1 M solution) and HCl (0.2 ml of 1 N solution) to the reflux chamber prior to the sample (1 ml). NO was then stripped from the sample by bubbling it with helium (12 ml·min^{-1} for 30 s) and detected by measuring the light emitted when NO and ozone (generated by electrostatic discharge within the NO Chemiluminescence Analyzer 270, Sievers Research, Boulder, CO, USA) interacted in front of a photomultiplier. Measurements were made at room temperature with an integration time of 2 s. A calibration curve was constructed using 10, 50, 100 and 500 µl of 10 ppm NO gas. The relationship between chemiluminescence (mV) and NO was: NO (pmol) = $-2.51+0.283 \times$ chemiluminescence (mV) [14]. The detection limit of the assay was <5 pmol. As HBSS, like other sources of water, contains NO_2^- [29], the endothelial NO+ NO_2^- values were corrected by subtracting the signal produced by a matching volume (1 ml) of HBSS which had not been exposed to the endothelium.

For each plate, $NO+NO_2^-$ accumulation in the perfusate over 10 min of perfusion was estimated by performing the $NO+NO_2^-$ assay before and after 10 min of perfusion. Ten plates were perfused with normoxic and 10 with hypoxic HBSS. To study the role of intracellular Ca^{2+} stores in the regulation of pulmonary artery endothelial NO production, additional measurements were performed in the presence of thapsigargin (10^{-6} M; n=4 normoxic and 4 hypoxic plates) and its solvent, dimethyl sulphoxide (DMSO) (n=2 normoxic and 2 hypoxic plates). Thapsigargin inhibits Ca^{2+} reuptake into a variety of intracellular stores [25]. NO production in response to Bk (10^{-8} M) was measured in five normoxic and five hypoxic plates. To exclude the possibility that increased $NO+NO_2^-$ levels in hypoxia were due to reduced NO inactivation by superoxide, the $NO+NO_2^-$ response to Bk was studied in the presence of superoxide dismutase (50 U·ml^{-1}). In addition, to see whether $NO+NO_2^-$ levels are increased in hypoxia even in the absence of flow and (presumably) shear stress, the perfusion of the plates was stopped in these experiments before the first HBSS sample was assayed for $NO+NO_2^-$. Bk was then injected and the second sample was analysed for $NO+NO_2^-$ 10 min later. During this 10 min period, the plates were not perfused. Due to the nitrogen atmosphere above the cells, the Po_2 of HBSS stayed at 4.9 kPa (37 mmHg) in the hypoxic plates.

Changes in either NO production or post-synthetic degradation and partition could theoretically account for the observed increase in $NO+NO_2^-$ amount in hypoxic plates. To distinguish between these two possibilities, we performed additional experiment in the same system but with no cells. $NO+NO_2^-$ concentration in HBSS was measured before and 10 min after 25 µl of saturated NO solution (2 mM) was injected into HBSS in the perfused plate. Three normoxic and three hypoxic plates were studied.

Measurement of $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured using the Ca^{2+} sensitive fluorophore, fura 2. To load the cells with fura 2, the plates were first washed three times with HBSS. They were then incubated in standard media containing fura 2 penta-acetoxymethyl ester (4 mM) at 37°C for 40 min. The esterified form of fura 2 is used to enhance its entry into the cells. After three more washes with HBSS, the media was replaced with a media without fura 2 and the cells rested at 37°C for 20 min to allow for complete de-esterification of intracellular fura 2. The plates were again flushed three times with HBSS, filled with HBSS, covered with a loosely fitting lid, and placed on the heated microscope stage (37°C). The floor of each plate had a "cut-out" sealed by a glass coverslip to permit optimum optical transmission for fluorescence determination of $[Ca^{2+}]_i$.

The quantitative fluorescence microscopy system (PTI DeltaScan 1) was obtained from Photon Technology International (South Brunswick, NJ, USA). Details of the $[Ca^{2+}]_i$ measurement using fura 2 have been described and discussed previously [27, 30]. Briefly, the light emitted at 510 nm by the fura 2-loaded cells in response to stimulation with a monochromatic light (wavelength of 340 and 380 nm alternating at 60 Hz) was collected with an epifluorescence microscope and quantified using a photomultiplier. A 75 W, ozone-free xenon lamp served as a light source. Two bandpass filters were used to select only the desired wavelength (340 or 380 nm), and a rotating chopper disk alternated the path of the light between the two filters with a frequency of 60 Hz. Cumulated emission at 510 nm of all 5–10 cells in the microscope's viewing field was viewed using a 40× oil/ fluor objective of a Nikon Diaphot inverted epifluorescence microscope (Nikon Instrument Group, Garden City, NY, USA) and passed to the photomultiplier. The output of the photomultiplier was recorded and used for $[Ca^{2+}]_i$ calculations by computer software provided as part of the PTI DeltaScan 1 system.

$[Ca^{2+}]_i$ was calculated from the ratio (R) of light emitted upon stimulation with 340 and 380 nm using the equation of GRYNKIEWICZ *et al.* [31] as described previously [27]:

$$[Ca^{2+}]_i = Kd \times \frac{(R - Rmin)}{(Rmax - R)} \times \frac{sf_{380}}{sb_{380}}$$

The dissociation constant for fura 2 (Kd) in this system, determined as described previously [30], was 1.39×10^{-7} M, with a viscosity correction factor of 1.0. Maximal and minimal R were determined on each plate at the end of the experiment by first lysing the cells with the detergent Triton X-100 (0.2 µl), thus saturating fura 2 with extracellular Ca^{2+} (Rmax), and then chelating all free Ca^{2+} with 2 mM ethylene glycol tetra-acetic acid (EGTA) (Rmin). The ratio of emissions of the Ca^{2+} free and bound standards upon 380 nm stimulation (sf_{380}/sb_{380}) was 11.1 in our system [30].

Accuracy of the Ca^{2+} assay was confirmed by comparing nominal and measured values of standard Ca^{2+} solutions *in vitro* [30]. Standards containing incremental Ca^{2+} concentrations were prepared by precise stepwise replacement of aliquots of Ca^{2+} free (10 mM EGTA) with equal volumes of Ca^{2+} replete (10 mM $CaCl_2$) solution. They contained 1 µM of fura 2. Calcium concentration in these standards was calculated using the FreeCal 4.0 computer program by L.F. Brass, University of Pennsylvania, Philadelphia, PA, USA. For these *in vitro* measurements, Rmin and Rmax was R of 10 mM EGTA and 10 mM Ca^{2+} standards, respectively. The measured Ca^{2+} concentrations were fairly close to the calculated values for all standards [30], confirming that the equipment was properly calibrated and the Kd appropriate for our experimental setting.

Pulmonary artery endothelial $[Ca^{2+}]_i$ was measured during 4–5 min of normoxic perfusion, and the cells were then challenged with hypoxia for 3–5 min. To study the effect of hypoxia on agonist-stimulated $[Ca^{2+}]_i$ increase, Bk (10^{-8} M) was administered during normoxia (in 12 plates) or hypoxia (in 5 plates) (fig. 1, protocol I). To differentiate between intracellular Ca^{2+} stores *vs* Ca^{2+} entry in the observed increase in $[Ca^{2+}]_i$ during hypoxia, a variety of interventions were employed. Twelve plates were studied in a nominally Ca^{2+} free solution (fig. 1, protocol II). In Ca^{2+} replete experiments, drugs that deplete intracellular Ca^{2+} stores, ryanodine (10^{-4} M) and thapsigargin (10^{-6} M), were administered 5–8 min before the hypoxic challenge (fig. 1, protocol III). In addition, the effect of thapsigargin dose on $[Ca^{2+}]_i$ was measured in another four normoxic Ca^{2+} replete plates (in this case the plates were not perfused but rather filled with HBSS). Whilst thapsigargin inhibits intracellular Ca^{2+} reuptake [25], ryanodine promotes Ca^{2+} release from endoplasmic reticulum [26].

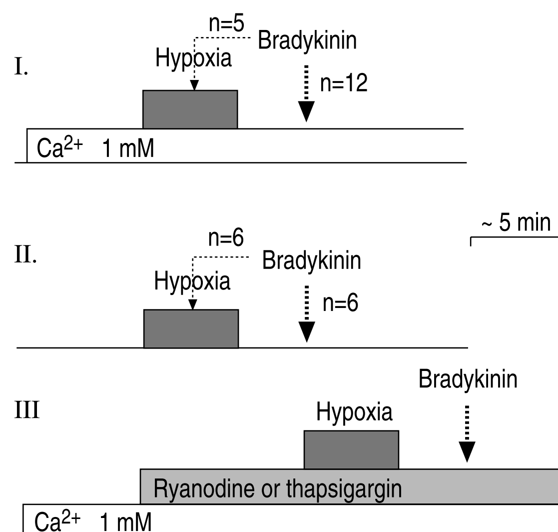

Fig. 1. – Schematic diagrams of $[Ca^{2+}]_i$ measurements. In protocol I (n=17 plates), $[Ca^{2+}]_i$ was measured in Ca^{2+} replete cells during normoxia, hypoxia, and bradykinin administration. In five plates bradykinin was given during, rather than after, hypoxia. Protocol II (n=12) was identical to protocol I except that extracellular Ca^{2+} was not added. Bradykinin was given during hypoxia in six plates. In protocol III (Ca^{2+} replete), ryanodine (n=7) or thapsigargin (n=4) (or its solvent, DMSO, n=2) were given in the bath before lowering Po_2. DMSO: dimethyl sulphoxide; Po_2: oxygen tension.

Drugs

Reagents were from Sigma Chemical Co., St. Louis, MO, USA except for NO gas from Matheson, Joliet, IL, USA, fura 2 AM from Molecular Probes, Eugene, OR, USA, ryanodine from Penick, Lyndhurst, NJ, USA, and thapsigargin from Calbiochem, La Jolla, CA, USA. They were dissolved in normal saline except for thapsigargin. Thapsigargin was first dissolved in DMSO (6.5 $mg \cdot ml^{-1}$), and serial dilutions of this stock solution with saline were then prepared, so that addition of 50 µl of these solutions into 50 ml perfusate (or 5 ml plate bath) resulted in thapsigargin concentrations of 10^{-6}–10^{-10} M whilst exposing cells to ≤0.01 % DMSO. This procedure was employed in order to keep DMSO concentration in the bath below the limit of 0.2%. DMSO concentrations higher than 0.2% may increase $[Ca^{2+}]_i$ [32]. All experiments with thapsigargin were paralleled by control experiments where DMSO without thapsigargin was given. NO gas was handled and saturated NO solution was prepared as described previously [33].

Statistics

Values are reported as the mean±SEM. Because of large data variance in some experiments, normal distribution was not presumed and nonparametric statistical tests were used. For comparisons between two groups, the Mann-Whitney U-test was used. When more then two groups were compared, the Kruskal-Wallis test was first calculated. If the p-value was less than 0.05, the Mann-Whitney U-test was then performed for the pair of groups with the largest difference in ranks. Wilcoxon signed rank test was used for paired comparisons. Calculations were performed using the StatView 4.01 computer program (Abacus Concepts, Berkeley, CA, USA). A value of p less than 0.05 was considered statistically significant.

Results

Nitric oxide release

The basal nitrite levels, present as contaminants in nonperfused normoxic and hypoxic aliquots of HBSS, tended to be lower in hypoxic than in normoxic HBSS (figs. 2 and 3). When HBSS was used to superfuse the cells, its $NO+NO_2^-$ content did not increase significantly in normoxia, but it did in hypoxia (fig. 2). Since there were no extraneous sources of NO_2^- in this study, $NO+NO_2^-$ accumulation reflected the rate of NO synthesis and/or degradation.

The increased amount of $NO+NO_2^-$ in the hypoxic superfusate was not due to a different post-synthetic degradation and/or partition of NO. When NO synthesis was excluded (by removing the cells from the system) and simulated by NO injection, the levels of $NO+NO_2^-$ in hypoxic plates equaled those in normoxic plates (fig. 3).

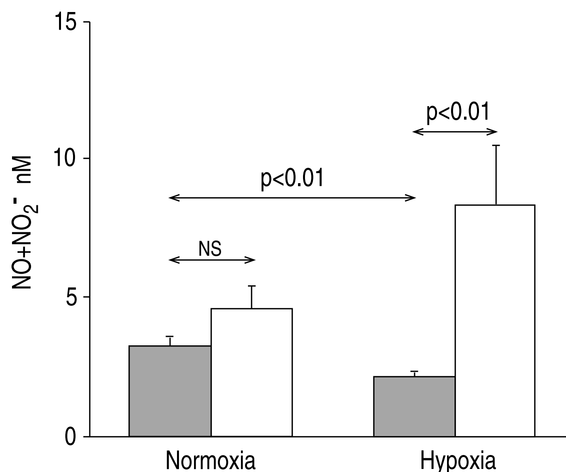

Fig. 2. – Hypoxia increases the accumulation of stable NO metabolite, NO_2^-, in the superfusate of pulmonary artery endothelial cells. The amount of $NO+NO_2^-$ in Hank's balanced salt solution (HBSS) was measured before and 10 min after HBSS was used to superfuse cultured cells, either in normoxia or hypoxia. Significant accumulation of $NO+NO_2^-$ in the superfusate of the cells occurred only in hypoxia. Data are presented as mean±SEM (n=10 per group). ▆: before superfusion of the cells; ▭: after 10 min of superfusion. NS: nonsignificant.

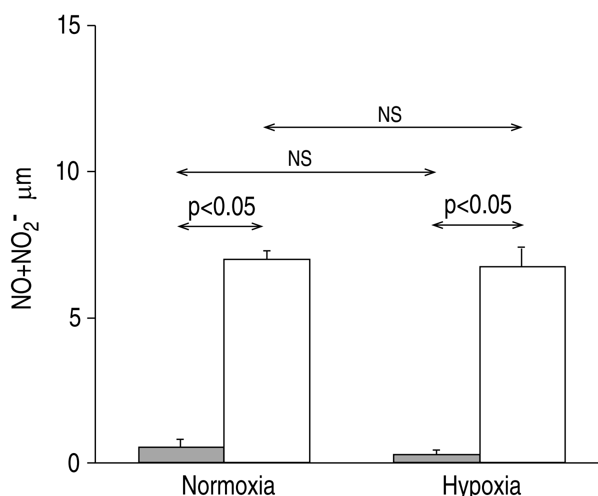

Fig. 3. – Moderate hypoxia did not alter the post-synthetic NO degradation/partition. The amount of $NO+NO_2^-$ was measured in the same system as in figure 2 but without the cells, before and 10 min after injection of 25 µl of saturated NO solution (2 mM). $NO+NO_2^-$ levels were elevated equally under hypoxic and normoxic conditions (by 6.4±1.2 and 6.4±0.7 µM, respectively). Data are presented as mean±SEM (n=3 per group). ▆: before NO; ▭: 10 min after NO. NS: nonsignificant.

In plates containing cells, hypoxia did not significantly change the $NO+NO_2^-$ levels in the presence of thapsigargin (19.2±10.7 nM in normoxia, 20.8±15.8 nM in hypoxia). However, thapsigargin did increase basal NO synthesis. The $NO+NO_2^-$ values were higher ($p<0.05$) in the presence of thapsigargin (20.0±8.8 nM; normoxic and hypoxic values pooled) than with its solvent (DMSO) alone (2.3±0.9 nM; normoxic and hypoxic values pooled).

Bk, administered in the presence of superoxide dismutase, increased $NO+NO_2^-$ levels more ($p<0.05$) in hypoxia (+47.3±12.2%) than in normoxia (+14.0±2.5%).

Intracellular calcium

With the addition of Ca^{2+} into the bath at the beginning of perfusion, $[Ca^{2+}]_i$ first rose quickly, and then it slowly equilibrated to a level significantly higher than that before Ca^{2+} was given (figs. 4 and 5). Drainage of the plate during the switches between normoxic and hypoxic perfusion (and *vice versa*) resulted in a transient, parallel increase both in 340 and 380 nm induced light emission, which did not alter the 340/380 nm ratio and, thus, had no effect on calculated $[Ca^{2+}]_i$.

Intracellular free calcium concentration increased rapidly with hypoxia (figs. 4 and 5). The hypoxic change in $[Ca^{2+}]_i$ appeared as a transient "spike", which returned to baseline within 40±6 s (fig. 4). This signal was not an artifact caused by the manipulation of perfusates, since an identical manipulation during the switch from hypoxic to normoxic superfusion had no effect on $[Ca^{2+}]_i$ (fig. 4). Hypoxia did not significantly diminish the Bk-induced increase in $[Ca^{2+}]_i$ (fig. 6).

When Ca^{2+} was not added to the baths, the measured levels of $[Ca^{2+}]_i$ were significantly reduced throughout the protocol (fig. 5). The magnitude of the hypoxic Ca^{2+} spike, however, was not reduced by low extracellular Ca^{2+} (table 1, figs. 5 and 6). On the other hand, the rise in $[Ca^{2+}]_i$ caused by Bk was reduced (table 1, figs. 5 and 6).

Under normoxic conditions, thapsigargin, unlike its solvent, caused a dose-dependent $[Ca^{2+}]_i$ peak (fig. 7) followed by a steady $[Ca^{2+}]_i$ level, which was slightly higher than before thapsigargin administration. Responses to hypoxia and Bk were significantly reduced by thapsigargin (table 1). Ryanodine also reduced both the

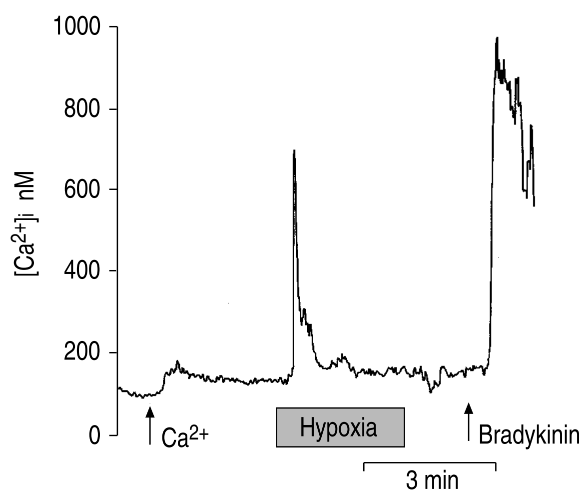

Fig. 4. – A representative trace of $[Ca^{2+}]_i$ from one plate of cultured pulmonary artery endothelial cells in the presence of 1 mM extracellular Ca^{2+}. Acute hypoxia transiently increases $[Ca^{2+}]_i$.

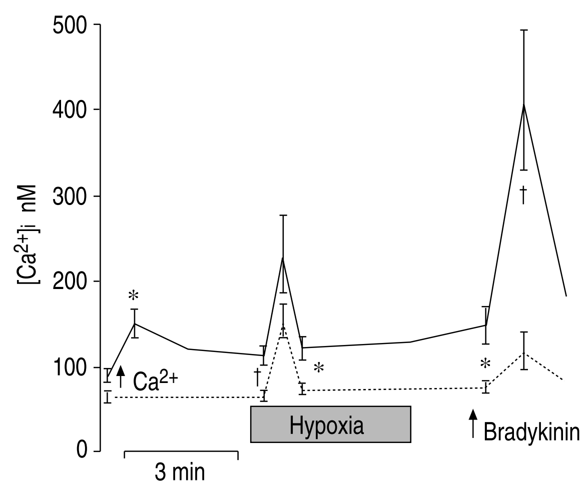

Fig. 5. – Reduced extracellular Ca^{2+} concentration diminishes $[Ca^{2+}]_i$ and its increase with bradykinin but not with hypoxia in pulmonary artery endothelial cells. Average timing of the peak responses to Ca^{2+}, hypoxia, and bradykinin is shown. Calcium (1 mM) was added to the perfusate in 17 plates but not in another 12 plates. The response to bradykinin is not shown for plates where bradykinin was given during hypoxia, but they were not significantly different from those shown here (252±58 nM in five Ca^{2+} replete and 110±19 nM in six Ca^{2+} free). Data are presented as mean±SEM. *: $p<0.05$ Ca^{2+} replete *vs* Ca^{2+} free; †: $p<0.01$ Ca^{2+} replete *vs* Ca^{2+} free. ———: Ca^{2+} replete; - - - - -: Ca^{2+} free.

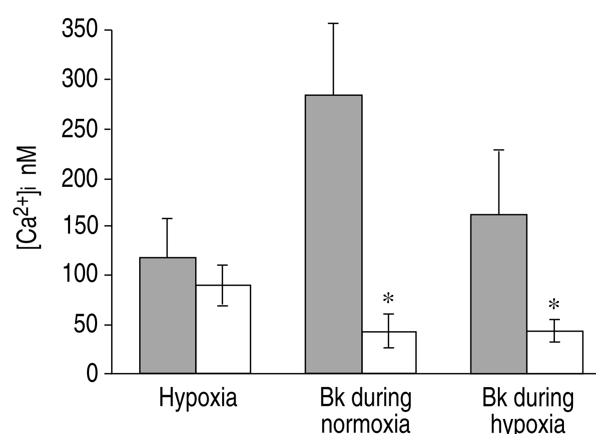

Fig. 6. – Extracellular Ca^{2+} depletion reduces the increase in pulmonary artery endothelial $[Ca^{2+}]_i$ in response to bradykinin (Bk) but not hypoxia. The vertical axis shows the peak change in $[Ca^{2+}]_i$ in response to the interventions listed along the horizontal axis. In 12 plates, no extracellular Ca^{2+} was added ("Ca^{2+} free"), whereas 1 mM Ca^{2+} was added to the perfusate in 17 plates ("Ca^{2+} replete"). Bradykinin was given during normoxia in 12 Ca^{2+} replete and six Ca^{2+} free plates, and during hypoxia in the rest of the plates. Data are presented as mean±SEM. *: $p<0.05$ Ca^{2+} free *vs* Ca^{2+} replete. ▒ : Ca^{2+} replete; □ : Ca^{2+} free.

Table 1. – Cytosolic calcium levels in bovine pulmonary artery endothelial cells

	Plates n	Basal nM	Hypoxia nM	Bradykinin nM
Control	12	116±12	257±55†	412±80†
Ca^{2+} free	6	69±6*	187±23†	117±21*
Thapsigargin	4	109±22	100±14*	107±23*
Ryanodine	7	85±7	116±12*	220±26†

Only those experiments where bradykinin (10^{-8} M) was given during normoxia are shown here. Data are presented as mean±SEM. Except for the "Ca^{2+} free" group, the experiments were perfor-med in the presence of 1 mM extracellular Ca^{2+}. Thapsigargin (10^{-6} M) or ryanodine (10^{-4} M) were given 5–10 min prior to the exposure to acute hypoxia. *: $p<0.05$ value differs from control; †: $p<0.05$ value differs from basal.

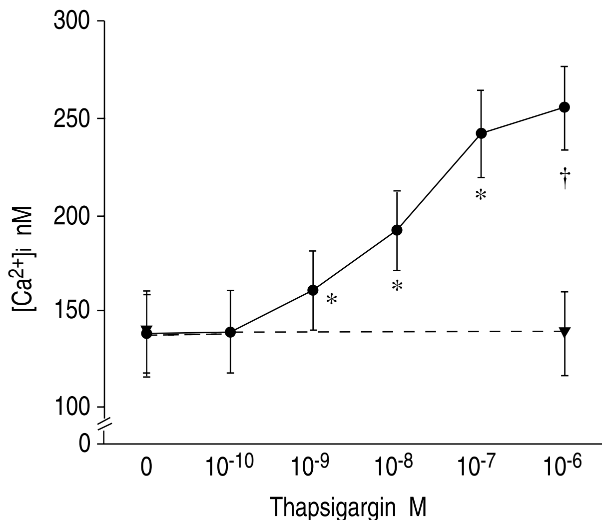

Fig. 7. – Thapsigargin causes dose-dependent increase in intracellular Ca^{2+} levels in pulmonary artery endothelial cells. Data are presented as mean±SEM (n=4 plates) of peak value achieved with each dose. Vehicle (DMSO) was given in the volume equal to that given with the highest thapsigargin dose. *: p<0.01 value differs from previous dose; †: p<0.05 value differs from previous dose. —●— : thapsigargin; ·····▼····· : vehicle. DMSO: dimethyl sulphoxide. Note that the vertical axis is cut-off from zero.

hypoxia- and Bk-induced rise in $[Ca^{2+}]_i$, although not as effectively as thapsigargin (table 1). Ryanodine did not increase normoxic $[Ca^{2+}]_i$.

Discussion

The objective of this study was to directly measure the effect of acute hypoxia on basal and Bk-evoked NO synthesis and its triggering mechanism, $[Ca^{2+}]_i$, in cultured pulmonary artery endothelial cells. The primary finding is that hypoxia elevates pulmonary endothelial NO synthesis and transiently increases $[Ca^{2+}]_i$. The hypoxic increase in $[Ca^{2+}]_i$ is not reduced when Ca^{2+} is omitted from the cells' bath but is prevented by pretreatment with drugs known to deplete intracellular Ca^{2+} stores, thapsigargin and ryanodine. Moreover, hypoxia does not inhibit the Bk-stimulated increase in endothelial $[Ca^{2+}]_i$, and enhances Bk-induced NO release. These data demonstrate that acute exposure to moderate hypoxia, independent of vasoconstriction, promotes NO synthesis. Furthermore, acute hypoxia does not interfere with the role of cytosolic Ca^{2+} as a second messenger in pulmonary artery endothelial cells.

NO synthase is a dioxygenase, which catalyses the reaction between molecular oxygen and L-arginine. Consequently, severe lack of oxygen (P_{O_2} <3.7 kPa (<28 mmHg)) may reduce NO synthase activity [34]. However, in intact cells the activity of the constitutive NO synthase is primarily regulated by $[Ca^{2+}]_i$ [20]. Indeed, when the production of NO by the pulmonary vasculature was measured in rats during chronic exposure to moderate hypoxia, it was increased, rather than reduced [14]. The current study advances our understanding of the mechanism by which hypoxia can elicit NO synthesis. In this *in vitro* model, hypoxia increased endothelial NO production independent of other cell types, shear stress, vasoconstriction, and superoxide ions.

This study attempted to use a degree of hypoxia which is relatively mild and could be tolerated *in vivo*. For example, healthy young men on Mt. Everest's summit (after several weeks of acclimatization) have arterial P_{O_2} levels of ~5.3 kPa (~40 mmHg) [35], which represents the limit of hypoxia survivable for a short time by humans. Most of the pulmonary endothelium, including that of relatively large arteries (≤2 mm) is exposed to alveolar, rather than venous, P_{O_2} [36]. The P_{O_2} of ~5.3 kPa (~40 mmHg) thus appears to be the lower limit of physiological hypoxia for the vessels most important in the regulation of pulmonary haemodynamics. The cells we used originate from the proximal pulmonary artery, where P_{O_2} is equal to central venous rather than alveolar P_{O_2}. However, they were grown in a 21% O_2 atmosphere for several weeks prior to the measurements, and had probably adapted to a higher P_{O_2}. For this reason, we choose P_{O_2} of 5.3 kPa (40 mmHg) as the hypoxic stimulus in this study.

WARREN *et al.* [16] measured relaxation of denuded, precontracted vascular rings superfused with effluent from cultured pulmonary artery endothelial cells which were exposed to Bk at varying degrees of hypoxia. They concluded that hypoxia impairs the release of endothelium-derived relaxing factor. However, in order to observe this they had to expose the cells to a near anoxic P_{O_2} of 2 kPa (15 mmHg) for at least 26 min. When they exposed the cells to a more physiological degree of hypoxia (P_{O_2} 5.6 kPa (42 mmHg)), the activity of endothelium-derived relaxing factor was not inhibited. NO was not measured in their study. In a more recent study, P_{O_2} of 2.7 kPa (20 mmHg) was found to increase $[Ca^{2+}]_i$ and guanylate cyclase activity (marker of NO synthesis) in human umbilical vein endothelial cells [37].

Two observations indicate that the transient increase in $[Ca^{2+}]_i$ caused by hypoxia in this study resulted from release of intracellular Ca^{2+}, probably from the endoplasmic reticulum (ER). Firstly, the hypoxic increase in $[Ca^{2+}]_i$ was unchanged in nominally Ca^{2+} free HBSS (table 1). Although we did not use a Ca^{2+} chelator to eliminate traces of extracellular Ca^{2+}, the significantly reduced response to Bk indicates effective Ca^{2+} removal.

A second piece of evidence for an intracellular source of the hypoxic $[Ca^{2+}]_i$ spike is its inhibition by thapsigargin and ryanodine. Thapsigargin is a high affinity inhibitor of intracellular Ca^{2+} transport adenosine triphosphatases (ATPases) [25]. Inhibition of these pumps inactivates the Ca^{2+} sequestering activity of the ER and depletes inositol-1,4,5-triphosphate-sensitive Ca^{2+} stores [25]. Consistent with this concept, thapsigargin increased the pulmonary endothelial $[Ca^{2+}]_i$ levels in this and in a previous study [24]. Ryanodine depletes ER Ca^{2+} stores by fixing the ER Ca^{2+} release channel in an open-subconductance state, which makes the ER leaky to Ca^{2+} [26]. The inhibitory effect of ryanodine also strongly supports involvement of the ER as a source of the hypoxic $[Ca^{2+}]_i$ spike. Thapsigargin and ryanodine have been shown to inhibit increases in $[Ca^{2+}]_i$ caused by acute hypoxia in pulmonary vascular myocytes [38–40].

STEVENS *et al.* [24] found that hypoxia ~5.3 kPa (~40 mmHg) reduced $[Ca^{2+}]_i$ in pulmonary artery endothelial cells after ~10 min [24]. We were interested in the immediate response of $[Ca^{2+}]_i$ to the change in P_{O_2}. On review of the data of STEVENS *et al.* (fig. 3 in [24]) there seems to be an initial increase in $[Ca^{2+}]_i$ similar to the current study. Only after 10–15 min of hypoxia did $[Ca^{2+}]_i$ decrease in their study. A composite picture of the effects of hypoxia on pulmonary endothelial $[Ca^{2+}]_i$ appears to include an initial transient increase, followed by a return to baseline, and later a decrease. In addition, our results provide evidence that the initial spike is caused by the Ca^{2+} release from intracellular stores, probably including ER, whereas STEVENS *et al.* [24] showed that the delayed $[Ca^{2+}]_i$ decrease appears to be related to the changes in the Ca^{2+} flux across the plasma membrane [24].

Although the relative importance of these contrasting effects of hypoxia on $[Ca^{2+}]_i$ for NO synthesis remains to be elucidated, the elevated NO production during the first 10 min of hypoxia substantiates the biological relevance of the transient increase in $[Ca^{2+}]_i$. This is further supported by the ability of thapsigargin to prevent both the hypoxic increases in $[Ca^{2+}]_i$ and $NO+NO_2^-$. Our observation of the marked ability of thapsigargin to increase NO synthesis agrees with the previously reported ability of thapsigargin to elicit endothelium-dependent vasorelaxation sensitive to inhibitors of NO synthesis [41, 42].

To study the possibility that increased NO_2^- accumulation was due to altered post-synthetic degradation and/or partition of NO in hypoxia, we measured $NO+NO_2^-$ levels in our system with variations in NO synthesis excluded. To do so, we replaced the variable cellular NO synthesis with an injection of an exact NO dose. The results (fig. 3) clearly show that under the conditions of our experiment the degradation/partition of NO did not differ between normoxia and hypoxia. Furthermore, the remote possibility that hypoxic solutions contain more $NO+NO_2^-$ than normoxic solutions was excluded by measurements of HBSS during normoxia and hypoxia before it was used for perfusion of the cells (figs. 2 and 3).

Hypoxia reduces pulmonary synthesis of oxygen-derived free radicals [43]. One of them, superoxide ion, is known to rapidly inactivate NO by forming peroxynitrite [44]. One possible explanation for higher $NO+NO_2^-$ levels in hypoxia might, thus, be that cultured endothelial cells produced less superoxide in hypoxia than in normoxia, and so less superoxide was available during hypoxia to inactivate NO. To study this possibility, we added superoxide dismutase to HBSS in the experiments with Bk. When the influence of superoxide was excluded by the presence of superoxide dismutase, the $NO+NO_2^-$ levels were still higher in hypoxia than in normoxia, indicating that alterations in superoxide production were not responsible for increased $NO+NO_2^-$ levels in hypoxia. Taken together, these experiments show that the increased $NO+NO_2^-$ accumulation in hypoxia was due to increased NO synthesis.

Acute hypoxia did not interfere with NO synthesis and $[Ca^{2+}]_i$ transients caused by Bk, a peptide which causes NO synthesis by binding a receptor and increasing $[Ca^{2+}]_i$. Bk increases $[Ca^{2+}]_i$, in part, by releasing intracellular Ca^{2+} but this effect is reinforced by entry of extracellular Ca^{2+} [22, 23, 27, 45]. In the current experiment, the most obvious difference in the endothelial $[Ca^{2+}]_i$ response to hypoxia and Bk was the partial dependence of Bk on extracellular Ca^{2+}, contrasted with the independence of hypoxia. Bk-induced increases in $[Ca^{2+}]_i$ were completely eliminated by thapsigargin. The fact that extracellular Ca^{2+} enhances the Bk response and yet the Bk response is inhibited by thapsigargin suggests that Bk may cause Ca^{2+} mediated Ca^{2+} entry (*e.g.* an initial release of intracellular Ca^{2+} promotes influx of Ca^{2+} across the plasma membrane). The results show that acute hypoxia does not block the signal transduction necessary for NO synthesis in the pulmonary artery endothelium.

In conclusion, hypoxia causes a transient increase in pulmonary artery endothelial $[Ca^{2+}]_i$, which is due primarily to the release of Ca^{2+} from intracellular stores. The same level of hypoxia (~5.3 kPa (~40 mmHg)) does not inhibit Bk-induced increase in $[Ca^{2+}]_i$. A physiologically relevant degree of hypoxia promotes NO synthesis by the pulmonary artery endothelium.

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References

1. Madden JA, Vadula Ms, Kurup VP. Effects of hypoxia and other vasoactive agents on pulmonary and cerebral artery smooth muscle cells. *Am J Physiol* 1992; 263: L384–L393.
2. Archer SL, Tolins JP, Raij L, Weir EK. Hypoxic pulmonary vasoconstriction is enhanced by inhibition of the synthesis of an endothelium-derived relaxing factor. *Biochem Biophys Res Commun* 1989; 164: 1198–1205.
3. Persson MG, Gustafsson LE, Wiklund NP, Moncada S, Hedqvist P. Endogenous nitric oxide as a probable modulator of pulmonary circulation and hypoxic pressor response *in vivo*. *Acta Physiol Scand* 1990; 140: 449–457.
4. Robertson BE, Warren JB, Nye PCG. Inhibition of nitric oxide synthesis potentiates hypoxic vasoconstriction in isolated rat lungs. *Exp Physiol* 1990; 75: 255–257.
5. Hasunuma K, Yamaguchi T, Rodman DM, O'Brien RF, McMurtry IF. Effects of inhibitors of EDRF and EDHF on vasoreactivity of perfused rat lungs. *Am J Physiol* 1991; 260: L97–L104.
6. Liu S, Crawley DE, Barnes PJ, Evans TW. Endothelium-derived relaxing factor inhibits hypoxic pulmonary vasoconstriction in rats. *Am Rev Respir Dis* 1991; 143: 32–37.
7. Sprague RS, Thiemermann C, Vane JR. Endogenous endothelium-derived relaxing factor opposes hypoxic pulmonary vasoconstriction and supports blood flow to hypoxic alveoli in anesthetized rabbits. *Proc Natl Acad Sci USA* 1992; 89: 8711–8715.
8. Barer G, Emery C, Stewart A, Bee D, Howard P. Endothelial control of the pulmonary circulation in normal and chronically hypoxic rats. *J Physiol* 1993; 463: 1–16.
9. McCormack DG, Paterson NAM. Loss of hypoxic pulmonary vasoconstriction in chronic pneumonia is not mediated by nitric oxide. *Am J Physiol* 1993; 265: H1523–H1528.

10. Oka M, Hasunuma K, Webb SA, Stelzner TJ, Rodman DM, McMurtry IF. EDRF suppresses an unidentified vasoconstrictor mechanism in hypertensive rat lungs. *Am J Physiol* 1993; 264: L587–L597.
11. Zhao L, Crawley DE, Hughes JMB, Evans TW, Winter RJD. Endothelium-derived relaxing factor activity in rat lung during hypoxic pulmonary vascular remodeling. *J Appl Physiol* 1993; 74: 1061–1065.
12. Dumas JP, Dumas M, Sgro C, Advenier C, Giudicelli JF. Effects of two K^+ channel openers, aprikalim and pinacidil, on hypoxic pulmonary vasoconstriction. *Eur J Pharmacol* 1994; 263: 17–23.
13. Feng CJ, Cheng DY, Kaye AD, Kadowitz PJ, Nossaman BD. Influence of Nω-nitro-L-arginine methyl ester, LY83583, glybenclamide and L158809 on pulmonary circulation. *Eur J Pharmacol* 1994; 263: 133–140.
14. Isaacson TC, Hampl V, Weir EK, Nelson DP, Archer SL. Increased endothelium-derived nitric oxide in hypertensive pulmonary circulation of chronically hypoxic rats. *J Appl Physiol* 1994; 76: 933–940.
15. Johns RA, Linden JM, Peach MJ. Endothelium-dependent relaxation and cyclic GMP accumulation in rabbit pulmonary artery are selectively impaired by moderate hypoxia. *Circ Res* 1989; 65: 1508–1515.
16. Warren JB, Maltby NH, McCormack D, Barnes PJ. Pulmonary endothelium-derived relaxing factor is impaired in hypoxia. *Clin Sci* 1989; 77: 671–676.
17. Rodman DM, Yamaguchi T, Hasunuma K, O'Brien R, McMurtry IF. Effects of hypoxia on endothelium-dependent relaxation of rat pulmonary artery. *Am J Physiol* 1990; 258: L207–L214.
18. Demiryurek AT, Wadsworth RM, Kane KA, Peacock AJ. The role of endothelium in hypoxic constriction of human pulmonary artery rings. *Am Rev Respir Dis* 1993; 147: 283–290.
19. Shaul PW, Wells LB, Horning KM. Acute and prolonged hypoxia attenuate endothelial nitric oxide production in rat pulmonary arteries by different mechanisms. *J Cardiovasc Pharmacol* 1993; 22: 819–827.
20. Nathan C. Nitric oxide as a secretory product of mammalian cells. *FASEB J* 1992; 6: 3051–3064.
21. Johns A, Lategan TW, Lodge NJ, Ryan US, vanBreeman C, Adams DJ. Calcium entry through receptor operated channels in bovine pulmonary artery endothelial cells. *Tissue Cell* 1987; 19: 733–745.
22. Peach MJ, Singer HA, Izzo NJ, Loeb AL. Role of calcium in endothelium-dependent relaxation of arterial smooth muscle. *Am J Cardiol* 1987; 59: A35–A43.
23. Luckhoff A, Pohl U, Mulsch A, Busse R. Differential role of extra- and intracellular calcium in the release of EDRF and prostacyclin from cultured endothelial cells. *Br J Pharmacol* 1988; 95: 189–196.
24. Stevens T, Cornfield DN, McMurtry IF, Rodman DM. Acute reductions in Po_2 depolarize pulmonary artery endothelial cells and decrease $[Ca^{2+}]_i$. *Am J Physiol* 1994; 266: H1416–H1421.
25. Inesi G, Sagara Y. Thapsigargin, a high affinity and global inhibitor of intracellular Ca^{2+} transport ATPases. *Arch Biochem Biophys* 1992; 298: 313–317.
26. Meissner G, el-Hashem A. Ryanodine as a functional probe of the skeletal muscle sarcoplasmic reticulum Ca^{2+} release channel. *Mol Cell Biochem* 1992; 114: 119–123.
27. Archer SL, Cowan NJ. Measurement of endothelial cytosolic calcium concentration and nitric oxide production reveals discrete mechanisms of endothelium-dependent pulmonary vasodilation. *Circ Res* 1991; 68: 1569–1581.
28. Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci USA* 1993; 90: 8103–8107.
29. Archer SL, Shultz PJ, Warren JB, Hampl V, DeMaster EG. Preparation of standards and measurements of nitric oxide, nitroxyl and related oxidation products. *Methods Enzymol* 1995; (in press).
30. Archer SL, Hampl V, Huang J, Cowan N. The importance of calcium in the regulation of EDRF synthesis in the pulmonary vasculature. *In*: Weir EK, Hume JR, Reeves JT, eds. Ion Flux in Pulmonary Vascular Control. New York, Plenum, 1993; pp. 223–246.
31. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; 260: 3440–3450.
32. Morley P, Whitfield JF. The differentiation inducer, dimethyl sulfoxide, transiently increases intracellular calcium ion concentration in various cell types. *J Cell Physiol* 1993; 156: 219–225.
33. Archer S. Measurement of nitric oxide in biological models. *FASEB J* 1993; 7: 349–360.
34. Rengasamy A, Johns RA. Characterization of endothelium-derived relaxing factor/nitric oxide synthase from bovine cerebellum and mechanism of modulation by high and low oxygen tensions. *J Pharmacol Exp Ther* 1991; 259: 310–316.
35. Groves BM, Reeves JT, *et al.* Operation Everest II: elevated high-altitude pulmonary resistance unresponsive to oxygen. *J Appl Physiol* 1987; 63: 521–530.
36. Sobol BJ, Bottex G, Emirgil C, Gissen H. Gaseous diffusion from alveoli to pulmonary vessels of considerable size. *Circ Res* 1963; 13: 71–79.
37. Busse R, Mülsch A, Fleming I, Hecker M. Mechanisms of nitric oxide release from the vascular endothelium. *Circulation* 1993; 87: V18–V25.
38. Salvaterra CG, Goldman W. Acute hypoxia increases cytosolic calcium in cultured pulmonary arterial myocytes. *Am J Physiol* 1993; 264: L323–L328.
39. Vadula MS, Kleinman JG, Madden JA. Effect of hypoxia and norepinephrine on cytoplasmic free Ca^{2+} in pulmonary and cerebral arterial myocytes. *Am J Physiol* 1993; 265: L591–L597.
40. Cornfield DN, Stevens T, McMurtry IF, Abman SH, Rodman DM. Acute hypoxia causes membrane depolarization and calcium influx in fetal pulmonary artery smooth muscle cells. *Am J Physiol* 1994; 266: L469–L475.
41. Matsuyama S, Shuntoh H, Katayama S, Tanaka C. Thapsigargin induces an endothelium-dependent, intracellular calcium ion-dependent vasodilation *in vitro*. *Life Sci* 1993; 53: 681–688.
42. Moritoki H, Hisayama T, Kondoh W, Takeuchi S. Thapsigargin, a Ca^{2+}-ATPase inhibitor, relaxes rat aorta *via* nitric oxide formation. *Life Sci* 1994; 54: PL153–PL158.
43. Archer SL, Nelson DP, Weir EK. Simultaneous measurement of oxygen radicals and pulmonary vascular reactivity in the isolated rat lung. *J Appl Physiol* 1989; 67: 1903–1911.
44. Freeman B. Free radical chemistry of nitric oxide: looking at the dark side. *Chest* 1994; 105: S79–S84.
45. Colden-Stanfield M, Schilling WP, Ritchie AK, Eskin SG, Navarro LT, Kunze DL. Bradykinin-induced increases in cytosolic calcium and ionic currents in cultured bovine aortic endothelial cells. *Circ Res* 1987; 61: 632–640.