Hypoxia Stimulates Prostacyclin Synthesis in Newborn Pulmonary Artery Endothelium by Increasing Cyclooxygenase-1 Protein

Amy J. North, Timothy S. Brannon, Lieselotte B. Wells, William B. Campbell, Philip W. Shaul

Abstract  In newborn lambs, pulmonary prostacyclin (PGI₂) production increases acutely in response to low oxygen. We tested the hypothesis that decreased oxygenation directly stimulates PGI₂ synthesis in arterial segments and cultured endothelial cells from newborn lamb intrapulmonary arteries. In segments studied at Po₂ of 680 mm Hg, the synthesis of PGI₂ exceeded prostaglandin E₂ (PGE₂) by 73%. Endothelium removal lowered PGI₂ by 77% and PGE₂ by 66%. At low oxygen tension (Po₂, 40 mm Hg), PGI₂ and PGE₂ synthesis rose by 96% and 102%, respectively. Similarly, in endothelial cells studied at Po₂ of 680 mm Hg, the synthesis of PGI₂ exceeded PGE₂ by 50%, and at low oxygen tension both PGI₂ and PGE₂ increased (89% and 64%, respectively). Endothelial cell PGI₂ synthesis maximally stimulated by bradykinin, A23187, or arachidonic acid was also increased at low Po₂ by 50%, 66%, and 48%, respectively. PGE₂ synthesis was similarly altered, increasing by 33%, 37%, and 41%, respectively. In contrast, lowering oxygen had minimal effect on PGI₁ and PGE₁ synthesis with exogenous PGH₂, which is the product of cyclooxygenase. Immunoblot analyses revealed that there was a 2.6-fold greater abundance of cyclooxygenase-1 protein at Po₂ of 40 versus 680 mm Hg, and the increase at lower oxygen tension was inhibited by cycloheximide. The cyclooxygenase-2 isozyme was not detected. Thus, attenuated oxygenation directly stimulates PGI₁ and PGE₁ synthesis in intrapulmonary arterial segments and endothelial cells from newborn lambs. This process is due to enhanced cyclooxygenase activity related to increased abundance of the cyclooxygenase-1 protein, and this effect may be due to increased synthesis of the enzyme protein. (Circ Res. 1994;75:33-40.)

Key Words: • cyclooxygenase • endothelium • hypoxic pulmonary hypertension • prostacyclin • prostaglandin E₂

Vasoconstriction

Vasoconstrictor prostaglandins such as prostacyclin (PGI₁) modify the pulmonary vasoconstrictor response to acute hypoxia in the newborn period.¹ ³ In vitro studies of perfused neonatal lamb lungs have demonstrated that an increase in pulmonary PGI₂ production occurs concomitant with the acute vasoconstriction.¹ In addition, in vivo experiments with inhibitors of prostaglandin synthesis indicate that the increase in PGI₁ production may serve as a compensatory mechanism to partially negate the constrictor effect of hypoxia.⁴ Furthermore, studies of lung homogenates from adult animals indicate that the effect of decreased oxygenation on PGI₁ production is independent of changes in intravascular pressure and shear stress.³ Since PGI₁ production in the lungs occurs primarily in the vascular endothelium,³ these observations suggest that acute changes in oxygenation may have a direct effect on PGI₁ synthesis in those cells.

To better understand the role of the endothelium in the enhancement of pulmonary PGI₁ production in response to acute hypoxia in the newborn, the present study was designed to evaluate the direct effect of oxygen on PGI₁ synthesis in vitro in intrapulmonary arterial segments and early-passage pulmonary artery endothelial cells from newborn lambs. Based on the in vivo and in vitro findings in whole lung,¹ ¹ ¹ ¹ the hypothesis was put forth that decreased oxygenation causes an acute increase in PGI₁ synthesis. In addition to testing this hypothesis, studies were performed to answer the following questions: (1) Is the synthesis of PGI₁ in relation to prostaglandin E₂ (PGE₂) comparable in intact intrapulmonary artery segments and early-passage endothelial cells? (2) Is PGE₂ synthesis also enhanced at lower levels of oxygenation? (3) At what step in the PGI₁ synthetic pathway is the effect of decreased oxygenation mediated?

Materials and Methods

Animal Model

Several groups of investigators have used the newborn lamb to assess the role of vasoconstrictor prostaglandins in oxygen modulation of vascular resistance in the developing pulmonary circulation.⁶ ¹ ⁰ As such, it is an excellent animal model for the in vitro study of the direct effects of oxygen on PGI₁ synthesis in newborn pulmonary artery endothelium. In the present investigation, intrapulmonary arteries and pulmonary artery endothelial cells were obtained from 15 mixed breed newborn lambs killed at 1 to 4 weeks of age. The lambs were born to multiparous ewes and were housed with their mothers in the Animal Resources Center of the University of Texas Southwestern Medical Center. The procedures followed in the care and euthanasia of the study animals were approved by the Institutional Review Board for Animal Research.

Arterial Segment Preparation

The lambs were euthanized with sodium pentobarbital (120 mg/kg) given intravenously. The lungs were immediately removed and placed in ice-cold phosphate-buffered saline (PBS; 0.01 mol/L PO₄, 0.15 mol/L NaCl, and pH 7.4). Further tissue preparation was performed in a cold room at 4°C as previously described.¹¹ The pulmonary arterial tree was rapidly dissected from the lung parenchyma and placed in fresh ice-cold PBS. Remaining fatty and connective tissue was gently removed, and the adventitia was grossly dissected from the arteries; care

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was taken not to disrupt the endothelium. Fourth-generation intrapulmonary arteries (0.5- to 1.0-mm external diameter) were isolated and placed in freshly prepared Krebs-Henseleit buffer gassed with 95% O₂/5% CO₂ at 37°C. The Krebs-Henseleit buffer contained (mmol/L) KCl 4.8, CaCl₂ 2.0, KH₂PO₄ 1.2, MgSO₄ 1.2, dextrose 11.0, NaCl 118, and NaHCO₃ 25 at pH 7.4. The generation of arteries studied was chosen in an effort to examine prostaglandin production in freshly obtained intact arterial segments as close to the level of the resistance vessels as possible. Segments with wet weights of 1 to 4 mg were cut, rinsed in fresh oxygenated Krebs-Henseleit buffer, and equilibrated in the oxygenated buffer at 37°C for 2 hours. In selected experiments, the endothelium was removed by repeated passage of knotted silk suture through the lumen of the arteries. The presence or absence of intact functional endothelium was confirmed in randomly chosen segments by (1) light microscopy of 3-μm sections of the arteries, (2) examinations of endothelium-dependent relaxation and (3) qualification of cGMP production with acetylcholine stimulation.

**Endothelial Cell Culture**

Intrapulmonary artery endothelial cells were harvested and maintained by methods modified from those of Johnson. Third-generation intrapulmonary arteries (1- to 3-mm external diameter) were isolated from the remainder of the pulmonary arterial tree under sterile conditions in a cold room at 4°C. Branch vessels were tied off with silk suture, and the arteries were placed in ice-cold M199 medium containing 2.5% iron-supplemented calf serum, 2.5% lamb serum, 1% antibiotic/antimycotic mixture, 0.15% nystatin, 0.15% gentamicin, and 0.10% tylosin. Further processing was performed at room temperature under a laminar flow hood. Remaining fatty and connective tissues were gently removed, with care taken not to disrupt the endothelium, and the arteries were flushed with M199 medium several times to remove cellular debris. The arteries were filled with a 0.2% to 0.4% solution of collagenase in M199 medium, tied off with silk suture, and incubated at 37°C for 30 minutes. The lumen contents were emptied into M199 medium containing 12.5% iron-supplemented calf serum, 12.5% lamb serum, 1% l-glutamine, 0.5% heparin, 1% antibiotic/antimycotic mixture, 0.15% nystatin, 0.15% gentamicin, and 0.15% tylosin, and the lumen was rinsed twice with M199 media.

The cells were pelleted, resuspended, and plated in gelatin-coated culture flasks, and they were propagated in RPMI medium containing 10% iron-supplemented calf serum and 10% lamb serum in a humidified incubator with 5% CO₂ in air at 37°C. The cells were studied at passages 1 through 4 in 24-well plates or 75-cm² flasks, near confluence. The identity of the endothelial cells was confirmed by phenotype (cobblestone appearance and contact inhibition), by immunofluorescence studies with antibody to factor VIII antigen, and by examinations of acetylated low-density lipoprotein uptake.

**Incubations for Prostaglandin Synthesis**

The incubation procedures used for the intrapulmonary arterial segments were similar to those we have used previously in studies of ovine fetal arteries. The segments were placed into sealed polypropylene chambers containing 2.0 mL of oxygenated Krebs-Henseleit buffer (PO₂, 680 mm Hg) at 37°C for 1 hour. After this preincubation period, the medium was replaced with fresh Krebs-Henseleit buffer, and 20-minute incubations were performed. At the end of the incubation, the medium was placed into ice-cold tubes containing 100 μg acetylsalicylic acid (ASA) and stored at −20°C until the time of assay for prostaglandins. The segments were placed in ice-cold 7% trichloroacetic acid and were stored at −20°C until protein content was determined by a modification of the method of Lowry et al. with bovine serum albumin as the standard. We have demonstrated in ovine fetal arteries that the prostanoids measured are newly synthesized and that synthesis is linear with time for at least 20 minutes. In all experiments, duplicate segments were studied from six or seven lambs.

The pulmonary endothelial cells grown in 24-well plates were preincubated for 15 minutes in room air with 1 mL of serum-free RPMI medium added per well. The preincubation medium was replaced with fresh serum-free RPMI medium, and 15-minute incubations were performed. At the end of the incubation, the medium was placed into ice-cold tubes containing 100 μg ASA and stored at −20°C until the time of assay for prostaglandins. The plates were air-dried and stored at −20°C until cell protein content was determined by the methods described above.

To assess oxygen modulation of prostaglandin synthesis, the incubations were carried out at three levels of in vitro oxygenation. The incubation medium was bubbled with one of three gas mixtures both before and after placement into the chambers containing the preincubated arterial segments or the wells of endothelial cells. The chambers and wells were sealed and vented while the media was gassed during the incubations. The resulting pH, PCO₂, and PO₂ in the medium were determined with an IL Micro Blood Gas Analyzer (Instrumentation Laboratory, Inc.). Gassing with 95% O₂/5% CO₂ yielded a PO₂ of 678 to 680 mm Hg, gassing with 20% O₂/5% CO₂/75% N₂ resulted in a PO₂ of 150 to 155 mm Hg, and the use of 0% O₂/5% CO₂/95% N₂ gave a PO₂ in the incubation medium of 40 to 42 mm Hg. The purpose of selecting these levels of PO₂ was to provide a wide spectrum of in vitro oxygenation and not to approach levels of oxygenation that yield cardiovascualr effects in vivo. The use of 5% CO₂ maintained the PCCO₂ of the incubation medium at 36 to 44 mm Hg and the pH at 7.35 to 7.4. Preliminary experiments using ethidium bromide and fluorescein diacetate revealed >95% cell viability after incubation at either PO₂ of 680 or 40 mm Hg for as long as 2 hours. Comparisons of PG₁ and PGE₂ synthesis between segments and cells were made at PO₂ of 680 mm Hg to ensure that prostaglandin synthesis was not limited by the availability of molecular oxygen required for cyclooxygenase (COX) activity, as in our previous studies.

In experiments designed to determine the reaction in the synthetic cascade that is involved in oxygen modulation of prostaglandin synthesis, selected wells of endothelial cells were incubated in RPMI medium alone, indicative of basal (nonstimulated) synthesis, and others were treated with agents that activate the synthetic pathways at various steps. Incubations were performed in the presence of bradykinin to assess the effects of oxygen on prostaglandin synthesis stimulated by receptor-mediated mobilization of arachidonic acid from phospholipids. Incubations with the calcium ionophore A23187 were performed to determine the effects of oxygen on prostaglandin synthesis stimulated by an increase in cytosolic free calcium, which activates arachidonic acid mobilization by a non–receptor-mediated process. Exogenous arachidonic acid was also used to stimulate prostaglandin synthesis to reveal if the effect of oxygen is reversed by provision of the precursor in excess. Reversal of the oxygen effect would be seen if the effect occurred proximal to arachidonic acid in the synthetic cascade, and a lack of reversal would indicate that the mechanism involves changes in COX and/or prostacyclin synthetase activity. Last, incubations were performed in the presence of exogenous prostaglandin H₂ (PGH₂), the product of COX activity and the substrate for prostacyclin synthetase, to differentiate between potential oxygen-mediated effects on the activities of these enzymes. Studies of concentration-related responses and time courses of activation of the synthetic cascade were performed preliminarily at PO₂ of 680 mm Hg. Maximal stimulation was found with bradykinin, A23187, arachidonic acid, and PGH₂ at 10⁻⁶ mol/L. As a result, this concentration was used for all agents in the experiments performed at varying levels of oxygenation. Basal and stimulated prostaglandin production were linear with time.
for at least 15 minutes; ensuing incubations were 15 minutes in duration. In all experiments, n = 4 to 6 for each determination, and findings were replicated in two or three studies with cells from different primary cultures.

Prostaglandin Assays

Samples of incubation medium were assayed for the stable metabolite of PGI₂, 6-ketoprostaglandin F₁₀ (6-keto-PGF₁₀), and for PGE₂ by radioimmunoassay as previously reported.²⁰,²² Briefly, the assay procedure used duplicate aliquots of standard (0 to 200 pg) and samples put into a mixture of Krebs-Henselitz solution and 0.1 mol/L phosphate-buffered saline plus 0.1% polyvinylpyrrolidone (1:1). Antiserum (0.1 mL; 1:4000 titer for 6-keto-PGF₁₀, and 1:14 000 titer for PGE₂) and 0.1 mL of [³H]6-keto-PGF₁₀ (12 000 disintegrations per minute [dpm]) or [³H]PGE₂ (16 500 dpm) were added, and the tubes were incubated at 4°C for 12 to 18 hours. Bound and free ligand were separated with dextran-coated charcoal, and bound ligand was counted by liquid scintillation spectrometry. The unknown quantities of prostaglandins were determined from the standard curves generated. The intra-assay and interassay coefficients of variation in the 6-keto-PGF₁₀ assay were 5.8% and 8.9%, respectively, at 250 pg/mL and 2.8% and 8.7%, respectively, at 1000 pg/mL. For PGE₂, they were 10.8% and 13.8%, respectively, at 250 pg/mL and 7.0% and 17.7%, respectively, at 1000 pg/mL.

Immunoblot Analysis for COX Protein

The effects of varying oxygenation on the abundance of COX protein were determined by immunoblot analysis. Pulmonary endothelial cells grown in 75-cm² flasks were preincubated for 15 minutes in room air at 37°C with serum-free RPMI medium. The preincubation medium was replaced with fresh serum-free RPMI medium gassed with either 95% O₂/5% CO₂ or 0% O₂/5% CO₂, and the gassing was continued during 5- to 30-minute incubations at 37°C. The cells were then harvested in ice-cold PBS, pelleted, resuspended in 50 mmol/L KH₂PO₄ buffer (pH 7.8) containing 250 mmol/L mannitol, 5 mmol/L diosodium EDTA, 0.1 mmol/L diethyldithiocarbamate, 0.1 mmol/L indomethacin, and 1% Tween 20, and ultrasonically disrupted (Branson Ultrasonics). The protein content of the preparation was determined by the method of Bradford using bovine serum albumin as the standard. In selected experiments, the cells were preincubated (120 minutes) and incubated in the presence of 0.5 μg/mL cycloheximide to differentiate between oxygen-mediated changes in COX protein synthesis and degradation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 7% acrylamide by the method of Laemmli,²⁶ and the proteins were electrophoretically transferred to nitrocellulose filters overnight. The nitrocellulose filters were blocked for 1 hour in buffer containing (mmol/L) NaCl 100, NaH₂PO₄ 20, and Na₂HPO₄ 80 (pH 7.5), with 1% Tween 20 and 5% dried milk, and then incubated with either 1:1000 COX-1 or 1:500 COX-2 antiserum for 2 hours at room temperature. COX-1 is purportedly the constitutive isozyme, and COX-2 is inducible and is thought to have a role in inflammatory processes.²⁷,²⁸ After incubation with primary antiserum, the nitrocellulose filters were washed in the 100 mmol/L NaCl buffer with 1% Tween 20 and incubated for 1 hour with a 1:10 000 dilution of anti-rabbit Ig antibody–horseradish peroxidase conjugate raised in donkey. The filters were washed in 100 mmol/L NaCl buffer with Tween 20, and the bands for COX were visualized by chemiluminescence and quantified by densitometry. Purified COX-1 and COX-2 proteins were used as positive controls.

Statistical Analysis

ANOVA with Newman-Keuls post hoc testing was used to compare mean values between more than two groups. Single comparisons between two groups were performed with non-paired Student’s t tests. Significance was accepted at P < .05. All results were expressed as mean±SEM.

Materials

6-Keto-PGF₁₀ standard was from Cayman Chemical Co, and PGE₂ was obtained from Upjohn. [³H]6-Keto-PGF₁₀ (120 to 180 Ci/mmol), [³H]PGE₂ (140 to 170 Ci/mmol), the anti-rabbit Ig antibody–horseradish peroxidase conjugate, and the ECL Western Blotting Analysis System used for chemiluminescence were from Amersham Corp. Bradykinin (acetate salt), A23187 (hemmagglutination salt), and arachidonic acid (sodium salt)
were obtained from Sigma Chemical Co. PGH$_2$ was from Biomol Research Laboratories, Inc; the hexane–ethyl acetate vehicle (10:1) was rapidly evaporated over N$_2$, and the compound was placed in RPMI buffer immediately before use. All solutions were prepared fresh daily. COX-1 and COX-2 antisera and purified COX-1 and COX-2 proteins were from Oxford Biomedical Research, Inc.

Results

Basal Prostaglandin Synthesis

Basal synthesis of PGI$_2$ compared with PGE$_2$ in intact intrapulmonary artery segments is shown in Fig 1A. In these experiments performed at P$_{O_2}$ of 680 mm Hg, PGI$_2$ production exceeded PGE$_2$ production by 73%. Similarly, in the endothelial cells, PGI$_2$ production exceeded PGE$_2$ production by 50% (Fig 1B). In separate experiments, removal of the endothelium from the arterial segments resulted in a decline in PGI$_2$ synthesis from 98±13 to 23±2 pg/mg protein per minute (P<.05) and a fall in PGE$_2$ synthesis from 55±4 to 19±1 pg/mg protein per minute (P<.05).

Stimulated Prostaglandin Synthesis in Pulmonary Endothelial Cells

Endothelial cell PGI$_2$ and PGE$_2$ production with stimulation of the synthetic cascade at various steps is shown in Fig 2. In these experiments performed at P$_{O_2}$ of 680 mm Hg, bradykinin caused a 2.8-fold increase in PGI$_2$ synthesis and a 2.3-fold increase in PGE$_2$ synthesis. Addition of the calcium ionophore A23187 also resulted in enhanced prostaglandin production, increasing PGI$_2$ and PGE$_2$ synthesis by 2.9-fold and 3.0-fold, respectively. Exogenous arachidonic acid caused a 5.8-fold increase in PGI$_2$ synthesis and a 3.6-fold increase in PGE$_2$ synthesis.

Effect of Oxygen on Basal Prostaglandin Synthesis

The effects of alterations in oxygenation on basal PGI$_2$ and PGE$_2$ production in both pulmonary arterial segments and endothelial cells are depicted in Fig 3. In the intact segments, PGI$_2$ synthesis rose 96% and PGE$_2$ synthesis rose 102% when oxygen tension was decreased. Similarly, in the endothelial cells, PGI$_2$ production increased 89% and PGE$_2$ production increased 64% at lower oxygen tension. The effect of decreasing oxygenation from P$_{O_2}$ of 680 mm Hg was evident in the segments at P$_{O_2}$ of 40 mm Hg and in the endothelial cells at P$_{O_2}$ of 150 mm Hg.

Effect of Oxygen on Stimulated Prostaglandin Synthesis in Pulmonary Endothelial Cells

To define the step in the biosynthetic cascade for PGI$_2$ that is modulated by oxygen, the effect of varying oxygenation on stimulated prostaglandin synthesis in pulmonary artery endothelial cells was determined. The effect of changes in oxygen tension on prostaglandin production maximally stimulated with bradykinin is shown in Fig 4. PGI$_2$ production with bradykinin rose an additional 82% when oxygenation was decreased from 680 to 150 mm Hg, paralleling the effect on basal (nonstimulated) PGI$_2$ synthesis (Fig 3). PGE$_2$
production with bradykinin also rose further when oxygen tension was decreased (38%). Fig 5 depicts the effect of changes in oxygenation on pulmonary artery endothelial cells maximally stimulated with A23187. When oxygen tension was lowered, there was an additional 124% increase in PGI₂ production with the calcium ionophore and a 61% increase in stimulated PGE₂ production. The effects of changes in oxygenation on prostaglandin production in the presence of excess arachidonic acid are shown in Fig 6. Similar to the observations in the previous two sets of experiments (Figs 4 and 5), there was increased production of both PGI₂ (54%) and PGE₂ (67%) at lower oxygen tension. A second series of experiments was performed to evaluate mechanisms distal to arachidonic acid in the synthetic cascade. The Table shows the results of studies in which pulmonary endothelial cell prostaglandin synthesis at P0₂ of 680 mm Hg was stimulated with arachidonic acid or PGH₂. With arachidonic acid, PGI₂ production increased 8.0-fold, and PGE₂ production increased 6.5-fold. These results are generally comparable to those reported in the first set of studies (Fig 2). In the presence of PGH₂, PGI₂ synthesis increased 9.6-fold, and PGE₂ production increased >500-fold.

Oxygen modulation of PGI₂ and PGE₂ synthesis was then examined in the presence of arachidonic acid or PGH₂ (Fig 7). With exogenous arachidonic acid present, PGI₂ synthesis increased 200% when oxygen tension was lowered (Fig 7A). In contrast, PGI₂ production in the presence of PGH₂ rose only 56% at lower oxygen tension. Similar results were obtained for PGE₂ (Fig 7B). With arachidonic acid, there was an increase in PGE₂ production of 126% when oxygenation was decreased. In contrast, with excess PGH₂ present, PGE₂ synthesis did not change when oxygen tension was decreased.

In the second series of experiments, concomitant determinations of basal and A23187-stimulated prostaglandin synthesis were also performed to confirm that the findings were similar to those obtained in the first set of studies. Basal production of both PGI₂ and PGE₂ was increased at lower levels of oxygenation, by 190% and 390%, respectively. Endothelial cells incubated with A23187 also showed a response to decreased oxygenation, with PGI₂ synthesis increasing 76% and PGE₂ synthesis increasing 99%. Although these findings were greater in magnitude, they were otherwise comparable to those depicted in Figs 3 and 5.

**Effect of Oxygen on COX Protein**

The effect of decreasing oxygenation on COX protein in the pulmonary endothelial cells is depicted in Fig 8. In the representative immunoblots shown (Fig 8A and 8B), COX-1 was detected in the cells and COX-2 was not. COX-1 protein abundance was greater at P0₂ of 40 versus 680 mm Hg. Quantitative densitometry from six independent experiments concurred with this observation, revealing that COX-1 protein increased 2.6-fold with decreased oxygenation (Fig 8C). The increase in COX-1 at lower oxygen tension was evident after 15 minutes, it persisted at 30 minutes, and it was inhibited by cycloheximide (n=3 experiments; data not shown).

![Fig 5](http://circres.ahajournals.org/)

**Fig 5.** Bar graphs showing the effect of varying oxygenation on prostaglandin I₂ (PGI₂) and prostaglandin E₂ (PGE₂) synthesis in A23187-stimulated pulmonary endothelial cells. Cells were incubated (15 minutes) in the presence of A23187 (10⁻⁵ mol/L) in medium with a P0₂ level of 680, 150, or 40 mm Hg. 6-Ketoprostaglandin F₁α and PGE₂ were measured by radioimmunoassay. Results are reported as percentage of synthesis with A23187 at P0₂ of 680 mm Hg. Values are mean±SEM (n=4 to 6). *P<.05 vs 680 mm Hg.

![Fig 6](http://circres.ahajournals.org/)

**Fig 6.** Bar graphs showing the effect of varying oxygenation on prostaglandin I₂ (PGI₂) and prostaglandin E₂ (PGE₂) synthesis in arachidonic acid–stimulated pulmonary endothelial cells. Cells were incubated (15 minutes) in the presence of arachidonic acid (10⁻⁶ mol/L) in medium with a P0₂ level of 680, 150, or 40 mm Hg. 6-Keto-prostaglandin F₁α and PGE₂ were measured by radioimmunoassay. Results are reported as percentage of synthesis with arachidonic acid at P0₂ of 680 mm Hg. Values are mean±SEM (n=4 to 6). *P<.05 vs 680 mm Hg.
Pulmonary Endothelial Cell Prostagacyclin and Prostaglandin E2 Synthesis Stimulated by Arachidonic Acid or Prostaglandin H2

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<tr>
<td>Basal</td>
<td>100±11%</td>
<td>100±20%</td>
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<tr>
<td>AA</td>
<td>796±47%*</td>
<td>652±50%*</td>
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<tr>
<td>PGH₂</td>
<td>963±105%*†</td>
<td>57 362±9885%*††</td>
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PGI₂ indicates prostacyclin; PGE₂, prostaglandin E₂; AA, arachidonic acid; and PGH₂, prostaglandin H₂. Values are mean±SEM. Cells were incubated at PO2 of 680 mm Hg in the presence of AA (10⁻⁵ mol/L) or PGH₂ (10⁻⁵ mol/L) for 15 minutes. 6-Ketoprostaglandin F₆α and PGE₂ were measured by radioimmunoassay. Results are reported as percent of basal (nonstimulated) production.

*P<.05 vs basal; †P<.05 vs AA; and ††P<.05 vs 6-ketoprostaglandin F₆α.

Discussion

In the present study, we determined the effect of acute changes in vitro oxygenation on prostaglandin synthesis in intact intrapulmonary arterial segments and endothelial cells from newborn lambs. We first validated the use of the early passage endothelial cells to examine this process in studies of basal PGI₁ and PGE₂ synthesis in segments and cells at one level of in vitro oxygenation (PO₂ of 680 mm Hg). We demonstrated that the majority of PGI₁ and PGE₂ synthesis in the segments is removed by deendothelialization (77% and 66% respectively). This suggests that PGI₁ and PGE₂ are primarily endothelial derived, particularly when the relative quantities of the different vascular cell types are considered, but it is also possible that an endothelial factor mediates prostaglandin production by the smooth muscle. PGI₁ production exceeded PGE₂ production by 73% in the intact segments. A comparable difference in PGI₁ and PGE₂ synthesis (50%) was found in the early-passage endothelial cells, indicating a conservation of phenotype in culture in regard to the mechanisms mediating relative PGI₁ versus PGE₂ synthesis. We then demonstrated that decreased oxygenation causes an acute increase in PGI₁ and PGE₂ synthesis in both the arterial segments and the endothelial cells. These findings indicate for the first time that oxygen directly modulates PGI₁ and PGE₂ synthesis in pulmonary artery endothelium in the newborn and that the early-passage cells provide an excellent model for further investigation of this process.

The results of the present studies are in agreement with observations made in whole lung, in that an acute decline in oxygenation increases PGI₁ production in perfused newborn lamb lung,¹ perfused adult rat lung,² and canine lung homogenate.³ In addition, inhibition of prostaglandin synthesis augments hypoxic pulmonary vasoconstriction in adult mammals of several species,¹ further suggesting that increased pulmonary prostaglandin synthesis occurs in the mature lung in response to hypoxia. Previous studies of pulmonary endothelial cells have involved more prolonged changes in oxygenation in experiments primarily with cells from large conduit arteries. Rabinovich et al²⁹ were unable to detect a change in PGI₁ or PGE₂ synthesis at lower oxygenation for 1 hour in endothelial cells cultured from the main pulmonary artery of newborn lambs. Microvascular endothelial cells from the same animals also failed to exhibit oxygen-mediated changes in prostaglandin production over 1 hour.²⁹ In contrast to our findings, Madden et al³⁰ reported decreased PGI₁ synthesis in adult bovine main pulmonary artery endothelial cells exposed to lower oxygen tension for 4 hours. In the present investigation, arterial segments from fourth-generation pulmonary arteries and cultured endothelial cells from the next most proximal generation were examined in an attempt to get as close to the level of the resistance vessels as possible. In addition, the acute effects of varying oxygenation were examined in an effort to mimic the rapid processes noted to occur in the intact newborn pulmonary circulation.¹ It is postulated that differences in the duration of varying oxygenation or in the generation of pulmonary artery studied may account for the disparities between the investigations. It should be reiterated that the purpose of varying oxygen tension in the present study was to examine PGI₁ and PGE₂ synthesis in vitro over a wide spectrum of oxygenation and not to mimic specific levels of oxygen tension that yield cardiovascular effects in vivo, particularly since oxygen tension is an insensitive indicator of oxygen availability in vivo, because it does not reflect the vast majority of blood oxygen content bound to hemoglobin.³¹

In addition to demonstrating an acute increase in pulmonary endothelial PGI₁ and PGE₂ synthesis at lower levels of oxygenation, we have identified the primary biosynthetic step involved in studies of prostaglandin production with activation of the synthetic pathway. First, we examined the effects of varying oxygenation on PGI₁ and PGE₂ synthesis in cells maximally stimulated with bradykinin, which acts via receptor-mediated processes.²³ The increase in PGI₁ and

![Fig 7. Bar graphs showing oxygen modulation of prostaglandin F₁α (PGF₁α) and prostaglandin E₂ (PGE₂) synthesis in the presence of arachidonic acid (AA) or prostaglandin H₂ (PGH₂). Pulmonary endothelial cells were incubated in the presence of AA (10⁻⁵ mol/L) or PGH₂ (10⁻⁵ mol/L) in medium with a PO₂ level of 680, 150, or 40 mm Hg. 6-Ketoprostaglandin F₆α and PGE₂ were measured by radioimmunoassay. Results are reported as percentage of synthesis with either AA or PGH₂ at PO₂ of 680 mm Hg. Values are mean±SEM (n=4 to 6). *P<.05 vs 680 mm Hg; †P<.05 vs 150 mm Hg.](http://circres.ahajournals.org/)}
PGE2 production seen at low oxygenation in the absence of exogenous stimulation (basal) was preserved in cells stimulated with bradykinin. These results indicate that the effect of oxygen is not due to changes in the production of a local receptor agonist activating arachidonic acid mobilization.

We next evaluated the stimulation of PGI2 and PGE2 synthesis by non-receptor-mediated activation of arachidonic acid mobilization by use of A23187. The degree of enhancement of basal PGI2 and PGE2 synthesis seen at low oxygenation was mimicked in cells maximally stimulated with A23187, indicating that the effect of oxygen on prostaglandin synthesis does not involve alterations in arachidonic acid mobilization. This conclusion is supported by the observation that cells incubated with excess arachidonic acid also exhibited increases in PGI2 and PGE2 production at low oxygenation that were comparable to the changes in basal synthesis. These findings indicate that the effect of oxygen on prostaglandin synthesis must be at the level of COX or that it involves alterations in PGI2 synthetase and PGE2-PG rh isomerase activity.

The possible effects of changes in oxygenation on the activities of these enzymes were differentiated by stimulating prostaglandin synthesis with exogenous PGH2, the product of COX and the substrate of PGI2 synthetase and PGE2-PG H isomerase.24 In these experiments, PGI2 synthesis activated by exogenous arachidonic acid rose 200% at decreased oxygen tension, whereas synthesis with exogenous PGH2 increased only 56%. In a similar manner, PGE2 synthesis with arachidonic acid rose 126%, whereas synthesis with PGH2 was unchanged. Thus, with stimulation of the synthetic pathway by the addition of the product of COX, there was a dramatic reduction in the acute effect of oxygen on PGI2 synthesis, and the effect on PGE2 synthesis was fully negated. These findings indicate that the major acute effect of oxygen on pulmonary endothelial prostaglandin synthesis is at the level of COX. However, an additional effect on PGI2 synthetase activity may also occur.

The activity of COX is highly dependent on the abundance of the enzyme protein because it undergoes self-catalyzed inactivation with a half-life of <10 minutes.32-34 In the present investigation, immunoblot analyses revealed that there is a 2.6-fold increase in COX-1 at lower oxygen tension, indicating that the hypoxia-induced enhancement in COX activity is related to greater abundance of that isoform of the enzyme. To our knowledge, this is the first study demonstrating that the abundance of COX-1 is modified by oxygen. COX-2 protein was not detected in the pulmonary endothelial cells examined at high levels of oxygenation, and it was also not induced by hypoxia. COX-1 and COX-2 are both expressed in endothelial cells, the former being purportedly constitutive in nature and the latter possibly involved in inflammatory processes.27,28 The hypoxia-related increase in COX-1 protein may be due to enhanced COX-1 protein synthesis, since it was inhibited by cycloheximide. Alterations in COX-1 degradation may also contribute to this effect.23 and further studies are needed to evaluate that process.

COX activity is also dependent on the availability of molecular oxygen, hydroperoxides, and heme.20,21 In the present study, PGI2 and PGE2 synthesis were increased at lower levels of oxygenation, indicating that oxygen has another effect on COX activity besides serving as a cofactor. Hydroperoxides are required for the initiation of COX activity, and thereafter, they paradoxically mediate the autoinactivation of the enzyme.35 As such, at low oxygen tension, which may yield decreased hydroperoxide levels, it is possible that there is less autoinactivation of COX. In addition, the availability of heme as a cofactor may be enhanced at lower levels of oxygenation. The contribution of alterations in the availability of these cofactors to the present observations in newborn pulmonary endothelial cells has not yet been determined.

The observations made in the present study contrast sharply with results we have reported for ovine fetal intrapulmonary arteries, in which there is attenuated PGI2 and PGE2 production at low oxygen tension in vitro.22 However, we have recently replicated this contrasting effect of oxygen before and after birth in concomitant studies of fetal and newborn arteries.36 We have demonstrated that the process in the fetal pulmonary endothelium is also mediated at the level of COX; potential changes in COX proteins in the fetal endothelium have not yet been examined.22 In addition, the oxygen-related alterations in PGI2 and PGE2 synthesis in the fetal or newborn group cause parallel changes in vascular smooth muscle cAMP production, indicating that there are resulting alterations in signal transduction pathways that modulate vasoconstrictor tone.36 We have previously proposed that direct oxygen modulation of pulmonary endothelial PGI2 and PGE2 synthesis at birth may be critically involved in the successful transition from fetal to newborn life.22 Soon thereafter, it appears that this mechanism is dramatically modified, such that it then serves as a compensatory process to negate the pulmonary vasoconstrictor effect of hypoxia in the newborn.

The findings in the present study may also have important physiological implications regarding oxygen-mediated vasoconstrictor responses in other vascular beds. There is evidence that the mature coronary circulation and the cerebral circulation of the newborn dilate with hypoxia and that these responses are at least partially

### Table

<table>
<thead>
<tr>
<th>COX</th>
<th>pO2</th>
<th>70kDa</th>
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<tbody>
<tr>
<td>A</td>
<td>1</td>
<td></td>
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<tr>
<td>B</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>680</td>
<td>40</td>
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**Fig 8.** A and B, Immunoblot analyses for cyclooxygenase (COX) in pulmonary endothelial cells incubated for 15 minutes in media with PO2 of 680 or 40 mm Hg. The antisemum used specifically recognizes either COX-1 (A) or COX-2 (B). The results are representative of six independent experiments. C, Bar graph summarizing data for the six experiments examining COX-1. Mean±SEM values are depicted for COX-1 abundance and expressed as the percentage of abundance at PO2 of 680 mm Hg. *P<.05 vs 680 mm Hg.
due to enhanced vasodilator prostaglandin synthesis.\textsuperscript{37-39} Considering the present results, it is postulated that hypoxia also increases COX-1 protein abundance in the endothelium of those vascular beds, thereby enhancing COX activity and PG\textsubscript{1} and PG\textsubscript{2} synthesis.

In summary, we have demonstrated that attenuated oxygenation directly stimulates PG\textsubscript{1} and PG\textsubscript{2} synthesis in intact intrapulmonary arterial segments and pulmonary endothelial cells from newborn lambs. This process is due primarily to enhanced COX activity related to increased COX-1 protein, and this effect may be due to increased synthesis of the enzyme protein. As such, oxygen-mediated changes in COX-1 expression may be important in the modulation of hypoxic pulmonary vasoconstriction during the newborn period.

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