Oxygen alters caveolin-1 and nitric oxide synthase-3 functions in ovine fetal and neonatal lung microvascular endothelial cells

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John, Theresa A., Basil O. Ibe, and J. Usha Raj. Oxygen alters caveolin-1 and nitric oxide synthase-3 functions in ovine fetal and neonatal lung microvascular endothelial cells. Am J Physiol Lung Cell Mol Physiol 291: L1079–L1093, 2006. First published September 22, 2006; doi:10.1152/ajplung.00526.2005.—We determined the effect of oxygen (~100 Torr (normoxia) and ~30–40 Torr (hypoxia)) on functions of endothelial nitric oxide (NO) synthase (NOS-3) and its negative regulator caveolin-1 in ovine fetal and neonatal lung microvascular endothelial cells (MVECs). Fetal NOS-3 activity, measured as NO production with 0.5–0.9 μM 4-amino-5-methylamino-2,7-difluorofluorescein, was decreased in hypoxia by 14.4% (P < 0.01), inhibitable by the NOS inhibitor N-nitro-L-arginine, and dependent on extracellular arginine. Caveolin function, assessed as FITC-BSA (160 μg/ml) endocytosis, was decreased in hypoxia by 13.5% in fetal and 22.8% in neonatal MVECs (P < 0.01). NOS-3 and caveolin-1 were physically associated, as demonstrated by communoprecipitation and colocalization, and functionally associated, as shown by cross-activation of endocytosis, by their specific antibodies and activation of NOS by albumin. Caveolin peptide, containing the sequence for the PKC phosphorylation site of caveolin, and caveolin antisera against the site increased NO production and endocytosis by 12.3% (P < 0.05) and 16% (P < 0.05), respectively, in normoxia and increased endocytosis by 25% (P < 0.001) in hypoxia. PMA decreased NO production in normoxia and hypoxia by 19.32% (P < 0.001) and 11.8% (P < 0.001) and decreased endocytosis in normoxia by 20.35% (P < 0.001). PKC kinase activity was oxygen sensitive, and threonine phosphorylation was enhanced in hypoxia. Pertussis toxin increased caveolar and NOS functions. These data support our hypothesis that increased PO2 at birth promotes dissociation of caveolin-1 and NOS-3, with an increase in their activities, and that PKC and an oxygen-sensitive cell surface G protein-coupled receptor regulate caveolin-1 and NOS-3 interactions in fetal and neonatal lung MVECs.

hypoxia; protein kinase C; G protein αi-subunit

ENDOTHELIAL NITRIC OXIDE SYNTHASE (NOS-3) is constitutively expressed in endothelial cells. Nitric oxide (NO) produced by the endothelium diffuses into the smooth muscle cell and causes vascular smooth muscle cell relaxation (12, 21) via stimulation of a soluble guanylate cyclase, cGMP accumulation, and cGMP-dependent protein kinase (PKG) activation (3, 15). In utero, in the hypoxic environment of the fetus, the pulmonary circulation is constricted and endogenous NO production is low. After birth, with oxygenation, endothelial NOS-3 activity and NO production increase, with resultant dilation of the pulmonary circulation (19). NOS-3 activity appears to be mainly controlled by posttranslational modifications, such as protein-protein interactions, subcellular localization in specialized compartments, and phosphorylation (23, 46). Importantly, NOS-3 binding with caveolin-1 by a specific protein-protein interaction targets NOS-3 into caveolae, rendering it inactive (25).

Caveolin-1 is the integral protein (48) of caveolae, which serve as transport vesicles (43). Caveolae mediate the fluid-phase endocytosis of macromolecules, e.g., albumin (24), a G protein-regulated function (39). Caveolin-1 is important in endocytosis and may play a significant role in physiological and pathological alveolar albumin and fluid clearance. It is not known whether caveolin-1 and NOS-3 act as signaling partners to promote the pulmonary vasodilation in fetal-to-neonatal transition.

In the present study, we have investigated the effects of oxygen on NOS-3 and caveolin-1 functions in pulmonary microvascular endothelial cells (MVECs) isolated from fetal and neonatal lambs. Figure 1 illustrates our proposed mechanism of interaction of caveolin-1 and NOS-3. Because PKC has been shown to inhibit NOS activity (20) and to be involved in hypoxic pulmonary vasoconstriction (4, 61), we studied the role of PKC in NOS-3 and caveolin functions. The involvement of a cell surface G protein-coupled receptor was studied using pertussis toxin (PTX), a G protein αi-subunit (Goi) inhibitor.

MATERIALS AND METHODS

Materials. Ketamine HCl and atropine sulfate were obtained from Phoenix; pentobarbital sodium from Virbac; endothelial cell growth factor E2759, FITC-BSA (catalog no. A9771), BSA, ovine serum albumin (OSA; catalog no. A6289), albumin blue 580 and dilution reagent (Fluka 05497 and Fluka 79438), dextrate, Na1, L-arginine HCl, N-nitro-L-arginine (L-NNA), mouse anti-NOS-3 antibody, rabbit anti-caveolin-1 antibody, rabbit anti-caveolin-1 Cy3 conjugate, cyclo-dextrin, SOD, and SOD-polyethylene glycol (PEG-SOD) from Sigma; PBS from Irvine Scientific; Gibco antibiotic-antimycotic mixture, dispase, DMEM, trypsin-EDTA, and NuPage 4–12% Bis-Tris gel from Invitrogen; FBS from Atlanta Biologicals; Triton X-100 from Fluka; donkey anti-rabbit Cy3 antibody conjugate, blood vessel-staining kit (catalog no. ECM590), including blocking reagent (catalog no. 90219), rins buffer (catalog no. 90218), and rabbit anti-von Willebrand factor (vWF; catalog no. 21540), and monoclonal mouse anti-CD31 (catalog no. 90219) from Chemicon; donkey anti-mouse Alexa 488 and goat anti-rabbit Alexa 568 secondary antibody conjugates, 4-amino-5-methylamino-2,7-difluorofluorescein (DAF-FM), DAF-FM diacetate, and 2,7-dichlorodihydrofluorescein diacetate (DCF) from Molecular Probes; triazolofluorescein (DAF-FM), DAF-FM diacetate, and 2,7-dichlorodihydrofluorescein diacetate (DCF) from Molecular Probes; trizolofluorescein (DAF-2T), DAF-2T diacetate, caveolin-1/3 control peptide, and caveolin-1/3 polyclonal antiserum from Cayman; sheep polyclonal anti-mouse

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L1079
**FETUS**

- **CAVEOLIN-1**
  - **ASSOCIATION**
  - **NOS-3**
  - **INACTIVE CAVEOLIN-1-NOS-3-COMPLEX**
  - **DISSOCIATION**
  - **NOS-3**

**NEONATE**

- **CAVEOLIN-1**
  - **ACTIVITY**

**HYPOXIA**

**NORMOXIA**

- **ENDOCYTOSIS**
- **NITRIC OXIDE SYNTHESIS**

physiological/pathological increased blood flow
alveolar protein/fluid clearance

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Fig. 1. Proposed scheme of effect of oxygen on caveolin-1 and nitric oxide synthase-3 (NOS-3). Caveolin-1-NOS-3 interaction is sensitive to PO2, with hypoxia facilitating their association and normoxia facilitating their dissociation. Measurement of functions of these two molecules in normoxia and hypoxia would reflect dynamics of their association and dissociation.

FITC-conjugated secondary antibody and monoclonal anti-caveolin-1 antibody from Abcam; rabbit anti-Arg-X-Tyr/Phe-X-pSer motif antibody and mouse anti-phosphothreonine antibody from Cell Signaling (Danvers, MA); PMA, PTX, and anti-eNOS monoclonal antibody from Cell Signaling; Goat anti-rabbit antibodies and mouse anti-phosphothreonine antibody from Cell Signaling; Alexa 568 (1:500 dilution) conjugates; then the slides were washed, mounted, and examined. **Passage 13** ovine fetal pulmonary vein smooth muscle cells (originally isolated in our laboratory) were similarly seeded and used as negative controls. Before experimentation, a sample of the endothelial cells was placed at normoxia and immunolabeled with monoclonal mouse anti-vWF antibody (150 μg antibody/10^10 cells) and sheep polyclonal anti-mouse FITC-conjugated secondary antibody and then passed through a fluorescence-activated cell sorter (FACS) analysis system (FACSCalibur, Becton Dickinson).

**Experimental conditions.** Confluent monolayers in multiple-well plates were derived from equal seeding of sibling cells (or the same passage) before passage in identical wells. Culture plates were placed in a Hepa-filtered incubator (Thermo Electron) at 37°C with 5% CO2 mixed in air. The cells were serum starved in DMEM for 2 h before experiments. The cells were subjected to hypoxia by replacement of the cell medium with medium that had been gassed with 2% O2-5% CO2-balance N2 for 10–15 min, so that medium PO2 was ~30–40 Torr. The culture wells were placed in the incubator, through which hypoxic gas flowed. For normoxia, a similar procedure with a gas mixture containing 21% O2-5% CO2-balance N2 was used. Fluorophores for measurement of endocytosis or NO production were added to the hypoxic or normoxic cells. The experiments were stopped on ice, and the cells were lysed for 5 min with lysis buffer at 31.25 μl/cm2 monolayer [50 mM Tris-HCL (buffer), 1% Triton X, and 0.5% SDS]. For fluorescence measurements, 40 μl of each lysate were diluted in 4 ml of water, vortexed briefly, and then passed through a luminescence spectrometer (model LS 30, Perkin Elmer). [Dilutions were adjusted to avoid autofluorescence interference (47).]

**Measurement of caveolin-1 function using endocytosis of FITC-BSA.** We used FITC-BSA endocytosis to assess caveolin-1 function. FITC-BSA, similar to albumin (24, 39, 51), is endocytosed by endothelial cells through caveolae. Because the time course of albumin uptake shows a steady rise and definite peak at ~15–30 min (24, 51), we chose a 15-min time point. Confluent MVEC monolayers from fetal and neonatal lamb lungs were separately serum starved and then incubated with 160 μg/ml FITC-BSA in PBS at 37°C for 15 min in normoxia or hypoxia. The experiments were stopped on ice, and the monolayers were washed with PBS and then with an acid buffer (0.2 M acetic acid and 0.5 M NaCl, pH 2.5) to strip off unabsorbed albumin and twice with PBS. The cells were lysed, and fluorescence of diluted lysates was measured. FITC fluorescence was detected at 494-nm excitation and 521-nm emission.

**Measurement of NOS-3 function using DAF-NO fluorescence.** We measured NO production with DAF to assess NOS-3 function. FITC-BSA, similar to albumin (24, 39, 51), is endocytosed by endothelial cells through caveolae. Because the time course of albumin uptake shows a steady rise and definite peak at ~15–30 min (24, 51), we chose a 15-min time point. Confluent MVEC monolayers from fetal and neonatal lamb lungs were separately serum starved and then incubated with 160 μg/ml FITC-BSA in PBS at 37°C for 15 min in normoxia or hypoxia. The experiments were stopped on ice, and the monolayers were washed with PBS and then with an acid buffer (0.2 M acetic acid and 0.5 M NaCl, pH 2.5) to strip off unabsorbed albumin and twice with PBS. The cells were lysed, and fluorescence of diluted lysates was measured. FITC fluorescence was detected at 494-nm excitation and 521-nm emission.

**Measurement of NOS-3 function using DAF-NO fluorescence.** We measured NO production with DAF to assess NOS-3 function. To study the relation between NO production and endocytosis, we measured basal NO production and endocytosis for 15 min. In experiments in which NO production alone was studied, measurements were extended to 45 min. Confluent MVECs from fetal and neonatal lamb lungs were separately incubated with DAF fluorophore in PBS. For measurement of NO in cell bathing medium, 0.78 μM DAF-2T or 0.5–0.78 μM DAF-FM was used; for measurement of intracellular...
NO from cell lysates, 0.93 μM DAF-2T diacetate or DAF-FM diacetate was used. Equivalents of fluorophore samples given to cell monolayers were measured for DAF autofluorescence, and any value obtained was subtracted from experimental values. Fluorescence of the adduct of DAF and NO was detected at 490- to 510-nm excitation and 485- to 535-nm emission. To confirm that changes in DAF fluorescence were due to constitutive NOS function, DAF fluorescence in the presence or absence of NOS inhibition was compared. Triplicate monolayers of newborn lamb MVECs were preincubated with the NOS inhibitor l-NNa (1 mM) in DMEM for 2 h before NO was measured with 0.5 μM DAF-FM. To determine whether the decrease in constitutive NO synthesis during hypoxia was dependent on arginine uptake, this experiment was repeated with neonatal MVECs that were serum starved and arginine deprived for 2 h in PBS (no amino acids), including a positive control in which 1 mM arginine was added to the cells.

Investigation of the role of caveolin-1 in activation of endocytosis and NOS function. Cy3-conjugated caveolin-1 antibody was added to control and endocytosing cells to determine whether expression of caveolin-1 increased during endocytosis. Triplicate monolayers of full-term fetal MVECs (passage 6) were serum starved for 2 h and then incubated with Cy3-conjugated rabbit anti-caveolin-1 antibody (1:10,000 dilution, wt/vol) with or without 100 μg/ml unlabeled albumin for 30 min. The experiment was terminated on ice, and the cells were subjected to acid stripping and washed and then lysed and measured spectrometrically for Cy3 labeling at 550-nm excitation and 570-nm emission.

To demonstrate that caveolin-1 and NOS-3 functions are interrelated, we used specific antibodies against caveolin-1 and NOS-3 and then measured endocytosis and NO production. To investigate the activation of NOS by its release from caveolin-1 increased during endocytosis. Triplicate monolayers of full-term fetal MVECs were serum starved for 2 h, washed, and subjected to normoxia or hypoxia for 30 min. During exposure to the same Po2, they were preincubated with monoclonal anti-caveolin-1 antibody (0.04 μg/ml) or monoclonal anti-NOS-3 antibody (0.57 μg/ml) or cyclodextrin (2 mM) for 2.5 h before addition of FITC-BSA (160 μg/ml) or DAF-FM (0.5 μM). Endocytosis or NO was measured for 25 min, and cell lysate FITC or cell medium DAF-NO fluorescence was determined as described above.

Investigation of physical and functional relations between caveolin-1 and NOS-3. To investigate a physical relation between caveolin-1 and NOS-3, we used the Sieze X protein G immunoprecipitation kit to coimmunoprecipitate NOS-3 with caveolin-1 antibody. According to the manufacturer’s instruction, a 50% slurry of immobilized protein G was prepared in a minicolumn. The gel was washed with the binding/wash buffer supplied with the kit, and 60 μg of caveolin-1 antibody were added to 200 μl of settled gel. After 15 min of binding, the gel was washed as prescribed by the manufacturer. To prevent coelution of the antibody with the antigen during the elution step, the bound antibody was cross-linked using 25 μl of disuccinyl disuberate cross-linker supplied with the kit. After the gel was washed first with elution buffer to remove any IgG that was not covalently coupled to the immobilized protein G and then with binding/wash buffer, antigen immunoprecipitation was performed.

Cell lysates from neonatal lung MVECs subjected to 15 min of hypoxia were used. Lysate diluted 1:1 with binding/wash buffer was loaded in the spin cup containing the gel and incubated for 1 h. The gel was centrifuged, and the flow-through lysate was saved for detection of any NOS not bound to the caveolin-1 on the gel. The gel was thoroughly washed repeatedly with binding/wash buffer as prescribed by the manufacturer and then loaded with ImmunoPure elution buffer. The first fraction was collected, and the elution was repeated on two more fractions. The fractions, the preelution flowthrough lysate, and the crude lysate were assessed by SDS-PAGE. Five microliters of sample buffer were added to 20 μl of each sample and 2 μl of 1 M DTT and boiled for 5 min. The samples were loaded on a NuPage 4–12% Bis-Tris gel and electrophoresed under 200 V for 5 min and then 180 V until the marker front reached the limit (~50 min). The separated proteins were transferred to a membrane under 30 V for 1 h. After the membrane was washed with 0.1% Tween 20-Tris-buffered saline and blocked with 5% nonfat milk, 3.3 μg/ml rabbit anti-caveolin-1 antibody and 3.3 μg/ml mouse anti-NOS-3 antibody were added for 1 h. After the membranes were washed three times, horseradish peroxidase-conjugated mouse and rabbit secondary antibodies were added for 1 h. Membrane development was carried out with Super Signal West Pico chemiluminescent substrate kit to prepare the developing solution, 15 s of membrane exposure to Blue Lite Autorad film, and film processing in a HOPE developer.

In another experiment, neonatal lung MVECs subjected to 15 min of hypoxia were coimmunostained for caveolin-1 and NOS-3 and examined by fluorescence microscopy using immunohistochemistry, as described above.

For investigation of a functional relation between caveolin-1 and NOS-3, the endocytosis experiments were modified for observation of simultaneous activation of NOS-3 and caveolin-1 function in the same confluent monolayers of neonatal lamb lung MVECs grown on Transwell culture plates with inserts. OSA (1 mM) was added to the upper chamber, and NO production in the upper chamber medium was measured using 0.78 μM DAF. OSA transcytosed from the upper chamber through the monolayer was measured simultaneously in the lower chamber samples by addition of 0.67 μM albumin blue 580 dye (27) and 1:100 dilution with water. Fluorescence of the adduct of albumin blue 580 dye and albumin was detected at 590-nm excitation and 620-nm emission.

Measurement of NO production after enhanced caveolin-1 activity. To investigate whether increased caveolin-1-associated endocytosis will facilitate dissociation of caveolin-1 and NOS-3 and increase NOS-3 activity, free albumin (100 μg/ml or 0.01 gram percent) was added to half of the newborn MVEC wells to enhance caveolar endocytosis immediately before addition of 0.78 μM DAF-2T for NO measurement. The other cell wells were used for control NO measurement. The cells were subjected to normoxia or hypoxia for 25 min during measurement of NO.

Investigation of the role of PKC in caveolar function. PKC is known to have specific effects on hypoxia related to pulmonary vasoreactivity (32, 35, 61). To determine whether phosphorylation of caveolin-1 by PKC is involved in regulation of caveolin-1 and NOS-3 functions, we preincubated experimental monolayers of newborn MVECs for 24 h with 45 μM PMA before NO or endocytosis measurements under normoxia or hypoxia. To further investigate the role of PKC on caveolin-1 and NOS-3 functions, we used caveolin-1/3-blocking peptide, which is derived from the rat caveolin-3 sequence. Caveolin-1 and caveolin-3 have 65% identical and 85% similar homology (56). The peptide amino acid sequence 19–41, CKEIDVNRDKDPKINIEDIVKVD, represents a conserved consensus site for phosphorylation by PKC (56, 57). We also used the antiseraum against the same sequence. In accordance with the manufacturer’s instructions, we incubated the peptide and antiseraum in a 1:1 (vol/vol) ratio for 1 h at room temperature and then added the mixture to the cell cultures. Final peptide concentration in the cell-bathing medium was 2 μg/ml with corresponding antiseraum volume. We preincubated the endothelial monolayers with the mixture for 2 h at 37°C and then observed its effect on endocytosis and NO synthesis in normoxia and hypoxia.

Investigation of PKC activity and serine/threonine phosphorylation of caveolin-1 in normoxia and hypoxia in newborn MVECs. For basal PKC activity measurements, confluent monolayers of newborn MVECs were subjected to 15 min of normoxia or hypoxia, as described above. The monolayers were then flash cooled and washed with ice-cold PBS, and the cells were lifted with cold isotonic lysis buffer. The cell suspensions were fractionated using the Active Motif nuclear extraction kit according to the manufacturer’s instructions.
Nuclear and cytosolic fractions stored at −80°C were subsequently analyzed for PKC activity using the Kinase-Glo Plus kit. The samples were analyzed as controls with no PKC-specific substrate and tests with the substrate (5 μg/ml). For all samples, the basic components of the reaction mixture were 7.5 mM ATP, 20 mM MgCl₂, 5 μg/ml substrate peptide, and 50 mM Tris. The reaction mixture volume was 80 μl + 20 μl of sample protein (10 or 20 μg of nuclear or cytosolic protein, respectively), and the reaction was allowed to proceed for 10 min at room temperature. Ten microliters of each of the terminated reaction mixture and Kinase-Glo Plus reagent were added to each well along with 130 μl of 50 mM Tris.

For observation of basal serine-threonine phosphorylation in MVECs in normoxia and hypoxia, confluent monolayers of newborn lamb MVECs were subjected to normoxia or hypoxia for 1 h. Routine immunohistochemistry described above was performed using a monoclonal anti-phosphothreonine or a rabbit anti-Arg-X-Tyr/Phe-X-pSer-motif (200 ng/ml) in combination with a rabbit or mouse anti-caveolin-1 primary antibody (1:350 and 200 ng/ml), respectively. The cells were counterstained with donkey anti-rabbit Cy3 and sheep anti-mouse FITC secondary antibody conjugates (3 μg/ml and 1:180, respectively).

Role of Gαi signaling in caveolin-1 and NOS-3 functions of lung MVECs. Gαi is thought to mediate caveolar signaling and albumin endocytosis (8, 39). To determine the role of Gαi signaling in caveolin-1 and NOS-3 functions and to determine whether PO2 affects this role, newborn MVECs were preincubated for 24 h with 50 μM PTX to inhibit Gαi before NO or endocytosis measurements under normoxia or hypoxia.

Investigation of involvement of reactive oxygen species in caveolin-1 signaling. To determine whether intracellular level of reactive oxygen species (ROS) changes with PO2, triplicate monolayers of newborn MVECs were incubated with DCF (5 μM) during 15 min of normoxia or hypoxia. Cultures were cooled on ice and washed three times with PBS, and DCF fluorescence was measured in cell lysates at 502-nm excitation and 523-nm emission.

To determine whether ROS affect endocytosis, we used PEG-SOD, which is readily cell permeable. SOD catalyzes the reduction of superoxide anions to hydrogen peroxide. To test whether intracellular superoxide and hydrogen peroxide have any effect on endocytosis, we incubated monolayers (n = 6) with or without PEG-SOD (700 ng/ml) for 15, 60, and 360 min under normoxia or hypoxia and added FITC-BSA for the final 15 min for endocytosis measurement.

Data analysis. Values are means ± SE. Data were analyzed by two-tailed Student’s t-test or paired t-test. Differences were considered statistically significant at P < 0.05.

RESULTS

Characteristics of cells used for study. The endothelial cell colonies showed pavement-form morphology at confluence (Fig. 2A, top). Immunohistochemistry revealed clear and uniform distribution of the endothelial cell markers vWF (with red Alexa 568) and CD31 (with green Alexa 488) colocalized in the cell body (orange). A higher CD31 intensity was present at cell-cell contact points (Fig. 2A, bottom). In confocal images, a small proportion (0.04) of cells was CD31 positive but lacked vWF. FACS analysis of the endothelial cells in suspension showed that the cells that reflected CD31-conjugated FITC staining were of one main population, with a normal distribution of ~600 forward-scatter units in the representation of fetal cells (Fig. 2Ba). This reflects a population with a normal distribution of smaller and larger cells. The side-scatter units (SSU) showed that the yield included a minor population of

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**A**

LUNG MICROVASCULAR ENDOTHELIAL CELLS

**B**

DEBRIS

NORMAL DISTRIBUTION

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Fig. 2. A: passage 3 microvascular endothelial cells (MVECs) derived from lung edge parenchyma. For cell isolation, sterile tissue mince was incubated with 2 U/ml dispase at 37°C under gentle agitation. Digest was processed through vacuum filtration, washed, centrifuged, and seeded in DMEM with 10% FBS, 1% penicillin-streptomycin, and 10 μl amphotericin B (Fungizone). Top: pavement form of endothelial cells at confluence. Bottom: cells incubated with anti-von Willebrand factor (vWF) and anti-CD31 for 2 h and counterlabeled with corresponding Alexa 568 and Alexa 488 secondary antibody conjugates for 1 h. Cells show vWF staining, CD31 staining, and colocalization of the two factors in the cells and intense CD31 expression at the cell-cell junctions (vWF + CD31). B: fluorescence activated cell sorter (FACS) analysis of endothelial cell culture. Cells were trypsinized, and suspensions were washed and attached with mouse anti-vWF antibody for 1 h, followed by rabbit anti-mouse FITC-conjugated secondary antibody for 1 h. Cells were washed through an FACS to measure uniformity of cell size by forward-scatter units (FSU) and granularity of cells by side-scatter units (SSU). Endothelial cells were mainly a single population with a normal distribution of cell size around an FSU peak of 560 units (a). Additional minor population of CD31-positive cells (<5% of total) represents some endothelial cells with lower SSU and FSU (b) and higher CD31 fluorescence intensity (c).
Hypoxia decreases NOS-3 function. Hypoxia decreased DAF-NO fluorescence in the fetal cell-bathing medium from the normoxic value of 57.4 ± 0.3 to 49.1 ± 0.6 (P < 0.001), indicating a decrease in NO production of 14.4% within 15 min. The hypoxic cell lysates had less DAF fluorescence (55.9 ± 0.7) than normoxic cell lysates (64.6 ± 0.8), indicating that hypoxia decreased NO production by 13.3% (P < 0.001). In newborn lamb cells used for comparison, hypoxia did not decrease NO production significantly in the cell lysates within the 15-min measurement period (from 123.167 ± 4.8 to 113.58 ± 0.8, a 7.7% decrease, P = 0.079) or in the cell-bathing medium (from 394.3 ± 6.5 to 379.7 ± 3.0, a 3.7% decrease, P = 0.18). These data (Table 1, Fig. 3) indicate that NOS-3 function is inhibited significantly by hypoxia in fetal lung MVECs.

In newborn cells in which NO measurement was extended to 45 min, NO production in hypoxic cells was 71.12% of that in normoxic cells, a significant decrease (P < 0.001). t-NNA inhibited NO production by 44.6% (P < 0.001) under normoxia and by 20% (P < 0.01) under hypoxia. NO production was decreased by t-NNA to 57.4% (P < 0.001) and 57% (P < 0.001) of normoxic values in the normoxic and hypoxic cells, respectively (Fig. 4). In cells that were arginine deprived in PBS, hypoxia did not decrease NO production and t-NNA treated hypoxic cells (Fig. 4).

Hypoxia decreases endocytosis. FITC-BSA fluorescence in the fetal cell lysates was 65.0 ± 1.6 in normoxia and 56.2 ± 0.9 in hypoxia, indicating that hypoxia decreased FITC-BSA endocytosis by 13.5% (P < 0.01; Table 1, Fig. 3). Hypoxia affected endocytosis more in newborn than in fetal cells; endocytosis was 49.6 ± 1.8 in normoxia and 38.2 ± 1.2 in hypoxia, a 22.8% decrease (P < 0.001; Table 1).

Table 1. Fetal and neonatal ovine lung MVEC responses in normoxia and hypoxia

<table>
<thead>
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<th>Activity</th>
<th>Normoxia</th>
<th>Hypoxia</th>
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<tr>
<td><strong>Fetal</strong></td>
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<tr>
<td>Endocytosis</td>
<td>65 ± 1.6</td>
<td>56.1 ± 0.9</td>
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<tr>
<td>NO production</td>
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<tr>
<td>Medium</td>
<td>57.4 ± 0.3</td>
<td>49.1 ± 0.1</td>
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<tr>
<td>Lysate</td>
<td>64.6 ± 0.8</td>
<td>55.9 ± 0.7</td>
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<td><strong>Neonatal</strong></td>
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<tr>
<td>Endocytosis</td>
<td>49.6 ± 1.8</td>
<td>38.2 ± 1.2</td>
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Values are means ± SE expressed as 4-amino-5-methylamino-2,7-difluorescein fluorescence. MVECs, microvascular endothelial cells; NO, nitric oxide.

Caveolin-1 antibody binding is upregulated during endocytosis. In full-term fetal cells preincubated with 100 μg/ml BSA, Cy3-anti-caveolin-1 immunolabeling was 319.92 ± 9.55, which was 50% greater than that of controls (212.74 ± 5.99, P < 0.01; Fig. 5A).

Fig. 3. Hypoxia attenuates endocytosis and nitric oxide (NO) release in fetal lamb lung MVECs. Confluent MVEC monolayers were serum starved for 2 h and then incubated with 160 μg/ml FITC-BSA for endocytosis measurement or 0.78 μM triazolofluorescein (DAF-2T) + 0.93 μM DAF-2T diacetate fluorophore for NO measurement at 37°C under normoxia (20% O2) or hypoxia (93% N2-5% CO2-2% O2). After 15 min, cell-bathing medium was sampled for DAF-NO fluorescence. Respective cell lysates were then measured for FITC-BSA or DAF-NO fluorescence. Values are means ± SE (n = 6–13). *P < 0.01; **P < 0.001 (Student’s t-test).

Fig. 4. NOS inhibition decreases NO production in normoxia and hypoxia: importance of extracellular arginine on hypoxia-sensitive NOS function. Confluent monolayers of newborn MVECs were preincubated with or without the NOS inhibitor N-nitro-l-arginine (t-NNA, 1 mM) for 2 h during serum starvation before NO measurements for 45 min under normoxia or hypoxia. DMEM [with extracellular arginine (84 mg/ml, 480 mM)], PBS (without extracellular arginine), or PBS + 1 mM l-arginine was used for serum starvation. 4-Amino-5-methylamino-2,7-difluorescein (DAF) fluorescence was measured at 495-nm excitation and 515-nm emission (λEX/EM). Values are means ± SE (n = 3–6). *P < 0.01; **P < 0.001 (Student’s t-test).
Activation and cross-activation of endocytosis by specific antibodies against caveolin-1 and NOS-3. In full-term fetal MVECs examined for the effect of specific antibodies, there was a 23% (P < 0.001) decrease in 25-min endocytosis of FITC-BSA in hypoxia (Fig. 5B) and a 10% decrease in NO production in hypoxia (Fig. 5C). Cyclodextrin decreased endocytosis by 11% (P < 0.05) in normoxia and by a further 6.5% in hypoxia (Fig. 5B). Cyclodextrin enhanced NO production by 19.33% in normoxia (P < 0.01) and by 6.98% in hypoxia (Fig. 5C). In these cells, preincubation with anticafeol-1 antibody (40 ng/ml) increased endocytosis in hypoxia from 216.22 ± 4.32 to 304.24 ± 11.8, a 40.7% increase (P < 0.001; Fig. 5B). Anti-caveolin-1 antibody had no effect on NO production in normoxia or hypoxia (Fig. 5C).

Anti-caveolin-1 antibody had no effect on NO production in normoxia or hypoxia (Fig. 5C). Anti-NOS-3 antibody (570 ng/ml) increased endocytosis in normoxia from 282.53 ± 12.82 to 437.5 ± 28.2, a 54.87% increase (P < 0.001), and also increased endocytosis in hypoxia from 216.22 ± 4.32 to 411.2 ± 19.1, a 90.18% increase (P < 0.0001). Anti-NOS-3 antibody had no effect on NO production in normoxia but increased NO production in hypoxia from 235.22 ± 7.1 to 260.65 ± 9.058, a 10.8% increase (Fig. 5C).

Coimmunoprecipitation and coexpression of caveolin-1 and NOS-3 and their functions measured in parallel in lung MVECs. In cell lysates from hypoxic newborn MVECs, caveolin-1 (22 kDa) and NOS (140 kDa) proteins were detected in eluents from the anti-caveolin-1 antibody-immobilized protein G column (Fig. 6A). The flow-through lysate unbound to the column as well as the crude cell lysate also consisted of these two proteins, as well as a ~160-kDa protein, which may represent NOS complexed with caveolin-1. Crude cell lysates further showed caveolin-1 bound to other proteins smaller than NOS.

In Transwell culture plates with confluent lamb MVEC monolayers where 1 mg/ml OSA was added to the upper chamber, NO and BSA accumulation increased closely in parallel from 15 min and over 3 h (Fig. 6B). Fluorescence microscopy of immunolabeled newborn lung MVECs subjected to 15 min of hypoxia showed caveolin-1 and NOS-3 distributed throughout the cell and colocalized in the cell membrane region (Fig. 6C).

Enhanced caveolin-1 activity increases NO production. We used 0.01 gram percent free albumin to activate endocytosis in serum-starved fetal lung MVECs and compared NO production...
with and without the albumin effect (Fig. 7). In these fetal cell cultures, hypoxia decreased DAF-NO fluorescence in the cell-bathing medium from 65.0 ± 1.6 to 56.2 ± 0.9, a decrease of 13.5%, and in the cell lysates from 64.6 ± 0.97 to 53.4 ± 0.78, a 17.3% decrease. DAF-NO fluorescence in cell lysates increased from 64.6 ± 0.97 to 72.25 ± 3 in normoxia after albumin-induced activation, an increase of 11.1% in NO production (P < 0.01). In hypoxia, the fluorescence values increased from 53.4 ± 0.78 in control cells to 64.25 ± 1.58 in albumin-activated cells, an increase of 20.32% (P < 0.001). DAF-NO fluorescence in the cell-bathing medium increased from 65.0 ± 1.6 to 72.25 ± 5.13 after albumin-induced activation in normoxia, an increase of 11.1% (P > 0.05). Under hypoxia, the values were 56.2 ± 0.98 in control cells and 62.1 ± 1.81 in albumin-activated cells, a small (10.48%) but significant increase (P < 0.01). These data show that albumin significantly increased intracellular NO production by 11.84% in normoxia and by 20.32% in hypoxia (P < 0.01, n = 9–13; Fig. 7).

PKC downregulates caveolin-1 and NOS-3 functions. DAF-NO fluorescence in the cell-bathing medium was 402.45 ± 5.19 in normoxic control, 324.7 ± 5.52 in normoxic PMA-treated, 379 ± 3.0 in hypoxic control, and 334.28 ± 5.30 in hypoxic PMA-treated cells. These data show that activation of PKC by PMA attenuated NO production by 19.32% in normoxia and 11.8% in hypoxia (Fig. 8, left). FITC-BSA fluorescence in cell lysates was 48.9 ± 1.94 in normoxic control, 38.95 ± 0.46 in normoxic PMA-treated, 37.92 ± 1.31 in hypoxic control, and 36.62 ± 1.02 in hypoxic PMA-treated cells. Thus PMA attenuated endocytosis in normoxia by

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Fig. 6. Physical and functional relation of caveolin-1 and NOS-3. **A**: NOS coimmunoprecipitated by caveolin-1 monoclonal antibody. Antibody was attached to a protein G column and cross-linked, then lysates from hypoxic cells were loaded. Eluents and crude cell lysates were subjected to SDS-PAGE, and separated proteins were analyzed by Western blotting using a cocktail of caveolin-1 and NOS-3 antibodies followed by corresponding horseradish peroxidase-conjugated secondary antibodies. Caveolin-bound NOS-3 coimmunoprecipitation (co-ip) shows a 140-kDa protein eluted from the caveolin antibody-bound column. Free NOS-3 and caveolin-1 represent unbound proteins in the flow-through lysate collected from the column before elution. Crude cell lysate shows that caveolin-1 binds to a continuum of proteins of varying molecular weight. **B**: parallelism of ovine serum albumin (OSA) transcytosis and NO release simultaneously measured from lamb MVEC monolayers in normoxia. Newborn lamb MVECs were grown on Transwell inserts. After serum starvation, 1 mM OSA was added to the upper chamber. At 15 and 210 min, NO in the upper chamber medium and OSA in the lower chamber medium were sampled with 0.78 μM DAF-FM and 0.67 μM albumin blue 580 indicators, respectively. Values are means ± SE for NO (n = 4) and OSA (n = 4) measured at 15 and 210 min, respectively. **C**: colocalization of caveolin-1 and NOS-3 in newborn lamb cells subjected to 15 min of hypoxia. For routine immunohistochemistry, cells were incubated with anti-caveolin-1 and anti-NOS-3 antibodies for 2 h and then counterlabeled for 1 h with species-corresponding secondary antibodies conjugated to Alexa 594 or FITC. Note some colocalization of NOS and caveolin-1 in the membrane.

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Fig. 7. Effect of albumin on intracellular NO production (A) and cell-bathing medium fluorescence (B) in normoxic and hypoxic cells. DAF-NO fluorescence in cell lysates increased from 64.6 ± 0.97 to 72.25 ± 3 in normoxia after albumin-induced activation, an increase of 11.84% (P < 0.01). In hypoxia, the values increased from 53.4 ± 0.78 to 64.25 ± 1.58, an increase of 20.32% (P < 0.001).
20.35%. There was no effect on endocytosis in hypoxic cells (Fig. 8, right).

The caveolin-1/3 peptide (with the sequence of the PKC phosphorylation site of caveolin) and corresponding antiserum increased NO production measured by DAF-NO fluorescence in the cell-bathing medium of normoxic cells by 12.3% (from 397.9 ± 14.9 to 447 ± 43, *P < 0.05) and had no further effect (376.3 ± 6.2) than hypoxia alone (379.7 ± 3.0) on hypoxic cells (Fig. 9, left; *n = 3–6). FITC-BSA fluorescence values obtained for endocytosis were 49.2 ± 2.05 for normoxic control and 57.75 ± 2.27 for caveolin peptide- and antiserum-treated cells and 38.92 ± 1.18 for hypoxic control and 47.67 ± 1.06 for caveolin peptide- and antiserum-treated cells. These data show that caveolin peptide and antiserum enhanced endocytosis by 16.3% in normoxia (*P < 0.05) and 25% in hypoxia (*P < 0.001; Fig. 9, right).

Differential basal PKC activity and serine/threonine phosphorylation in normoxic and hypoxic cells. In cell fractions obtained from newborn MVECs subjected to 15 min of normoxia or hypoxia, basal PKC activity measured as percent change in supplied ATP was 6.3% and 10.37% in nuclear fractions of normoxic and hypoxic cells and -11.26% and -4.48% in cytosolic fractions of normoxic and hypoxic cells, respectively (*n = 2; Fig. 10, top). Immunohistochemistry with anti-phosphothreonine-specific antibody showed intense phosphothreonine expression colocalized with caveolin-1 in the membranous regions in hypoxic cells and less intense intraganelle phosphothreonine expression in normoxic cells (Fig. 10, bottom). Serine phosphorylation was not captured with the motif-specific antibody.

Gαi involvement in caveolin-1 and NOS-3 functions in normoxia and hypoxia. Newborn lamb lung MVECs were preincubated with the Gαi inhibitor PTX (50 μg/ml). PTX enhanced DAF-NO fluorescence in the cell-bathing medium from 408.3 ± 9.7 in normoxia and from 384 ± 3.0 to 435.4 ± 19.15 in hypoxia. PTX similarly increased FITC-BSA fluorescence in cell lysates from 48.9 ± 1.88 to 56.18 ± 2.
1.4 in normoxia and from 38.14 ± 1.36 to 46.2 ± 0.8 in hypoxia. From these data, it appears that inhibition of Gαi increased NO production by 5.71% in normoxia and by 13.41% in hypoxia and also increased endocytosis by 14.89% in normoxia and by 21.13% in hypoxia (Fig. 11).

Involvement of ROS in caveolin-1 and NOS-3 functions. DCF fluorescence was 40% greater in hypoxic than in normoxic cells, suggesting a significant increase in ROS generation in hypoxia (P < 0.0001; Fig. 12A). Scavenging of superoxide with subsequent accumulation of hydrogen peroxide by the addition of PEG-SOD decreased endocytosis significantly at 1 h (Fig. 12B). PEG-SOD decreased endocytosis by 0.16%, 15%, and 11% in normoxia after 15, 60, and 360 min of exposure, respectively, and by 5.37%, 17.33%, and 5.87% in hypoxia. PEG-SOD significantly reduced endocytosis at 1 h [P = 0.0065 for normoxia and P = 0.036 for hypoxia (paired t-test), n = 6]. The difference between pairs, with and without PEG-SOD, was not significant at 15 and 360 min for normoxia and hypoxia.

**DISCUSSION**

The mechanism by which oxygen increases NOS function and, thereby, increases NO production at birth is not fully understood. In this study, we first determined the effects of oxygen on the functions of endothelial NOS (NOS-3) and its negative regulator caveolin-1. We then determined whether cross-activation of NOS-3 occurs when caveolin-1 function is activated to establish a functional relation between caveolin-1 and NOS-3 in normoxia and hypoxia. Finally, we examined the effects of PMA and PTX on NOS-3 and caveolin-1 functions to determine the roles of PKC and G proteins in caveolin-1 function.

We used MVECs, because in the lung these cells are closest to the air-blood interface. The isolated cells were identified as endothelial cells by morphology and by a well-distributed presence of vWF and CD31 (Fig. 2A). FACS analysis showed that the cells consist of a main population of cells with a normal distribution of cell size and granularity, as expected in a healthy culture (Fig. 2B). A minor population of CD31-positive cells (<5% of total) was less granular, with smaller SSU and more intense CD31-FITC fluorescence (Fig. 2B). These may be endothelial precursors or progenitors lodged in the microvasculature (59) before cell isolation, but we did not explore this speculation. The fetal and neonatal cells used for experiments were grown in normoxia. Because we observed that the effect of PO2 on endocytosis and NO production were similar (in the same direction) in fetal and neonatal cells (Table 1), we determined the effects of signal modulators mainly in neonatal cells.

Basal levels of NO production have been difficult to determine, because NO has a very short half-life of 3–6 s, and stimulated NO release is only ~500 ± 20 nmoI/s (7). DAF-FM forms a relatively photo-stable water-soluble adduct, a fluorescent benzotriazole, with NO (29), at concentrations as low as 3 nM and can trap NO as it is produced over the period of interest (15–45 min). Importantly, low doses of DAF pick up minute differences between groups with minimal DAF autofluorescence (47). Using DAF, we found that isolated endothelial cells...
in confluent monolayers synthesize NO under basal normoxic conditions and that this is attenuated by hypoxia (Fig. 3), consistent with our hypothesis. By comparing NO production in the presence and absence of the NOS inhibitor L-NNA and/or supplementation with arginine under normoxia or hypoxia, we ensured that NO produced by the cells was from constitutive enzyme activity. The $K_m$ of NOS for L-arginine is in the unit micromolar range (45); generally, circulating L-arginine and L-arginine concentration within endothelial cells is 100–800 $\mu$M (5, 18), so NOS is normally saturated with the substrate, and L-arginine is not rate limiting for the enzyme (11). In this study, the difference in NO production between hypoxia and normoxia appears to depend on arginine uptake and constitutive NOS activity (Fig. 4) and, therefore, may involve NOS enzyme complexed with the cationic amino acid transporter (that internalizes arginine) within caveolae (38). Hypoxia has been demonstrated to decrease arginine uptake in endothelial cells (5). However, from the present data, under hypoxia, it appears that NO is also produced from a source that may be less dependent on arginine uptake or even from

Fig. 10. Differential PKC activity and threonine phosphorylation in normoxia and hypoxia in newborn MVECs. For basal PKC activity measurements, newborn MVEC suspensions were fractionated using Active Motif nuclear extraction kit according to the manufacturer’s instructions. PKC kinase activity was analyzed in duplicate nuclear (10 $\mu$g) and cytosolic (20 $\mu$g) fractions for 10 min using the Kinase-Glo Plus kit with and without PKC-specific substrate. Reaction mixture contained 7.5 mM ATP, 20 mM MgCl$_2$, 5 $\mu$g/ml substrate peptide, and 50 mM Tris in double-distilled water. Kinase-Glo Plus reagent was added as specified, and luminescence was measured. Top: hypoxia increased PKC activity in the nuclear fraction and normoxia increased the activity in the cytosolic fraction. Middle and bottom: basal serine-threonine phosphorylation in confluent monolayers of newborn lamb MVECs subjected to 1 h of normoxia or hypoxia. Routine immunohistochemistry was performed using a monoclonal anti-phosphothreonine (200 ng/ml) and a rabbit anti-caveolin-1 antibody (200 ng/ml). Cells were counterstained with donkey anti-rabbit Cy3 and sheep anti-mouse FITC conjugates, respectively. Note phosphothreonine distribution in cells in normoxia and hypoxia. Hypoxia caused colocalization of caveolin-1 and phosphothreonine in the plasma membrane.
constitutive NOS activity. It is possible that, under hypoxic conditions, intracellular arginine pools (52) are utilized for subbasal synthesis of NO or nitrate compounds are converted to NO (18).

In parallel with NO measurements, we observed that the monolayers demonstrated endocytosis of FITC-BSA (Fig. 3) and transcytosis of albumin (Fig. 6). We have used endocytosis to measure caveolin-1 activity. On testing the relation between caveolin-1 activation and endocytosis activation, we showed that when a ligand such as BSA initiates the endocytotic process, caveolin-1 antibody binding detected by Cy3 labeling in live fetal lung MVECs is increased by 50% within 30 min (Fig. 5A). This may mean that there is increased recruitment of caveolin-1 or that caveolar changes during endocytosis increase the caveolin-1 exposure for antibody binding. In this study, the terms endocytosis and caveolin-1-activity are used interchangeably.

Endocytosis was attenuated by hypoxia (Fig. 3), consistent with our hypothesis. Small, but consistent, changes produced by hypoxia (Fig. 4) may indicate that the effect of extracellular

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**Fig. 11.** Inhibition of G protein αi-subunit (Gαi) by pertussis toxin (50 μg/ml, PTX) enhances endocytosis and NO production in newborn ovine lung MVECs. Cells were preincubated with PTX in PBS for 24 h. Monolayers were washed. Medium was replaced with FITC-BSA (160 μg/ml) for endocytosis cultures and DAF-2T (0.78 μM) for NO cultures. After 15 min of incubation at 35°C in hypoxia or normoxia, cell-bathing medium was sampled for NO or cells were washed and lysed, and cell lysates were measured for FITC-BSA fluorescence. Values are means ± SE (n = 3). *P < 0.01; **P < 0.001 (Student’s t-test).

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**Fig. 12.** Reactive oxygen species are increased in hypoxia and may have a role in caveolar regulation. To determine whether intracellular level of reactive oxygen species changes with Po2, triplicate monolayers of newborn MVECs were incubated with 5 μM 2,7-dichlorodihydrofluorescein diacetate (DCF) during 15 min of normoxia or hypoxia. Cultures were cooled on ice and washed three times with PBS, and cell lysates were measured for DCF fluorescence at 502-nm excitation and 523-nm emission. To test whether intracellular hydrogen peroxide accumulation has an effect on endocytosis, monolayers were incubated with (700 ng/ml) or without PEG-SOD (n = 6) for 15, 60, and 360 min under normoxia or hypoxia, and FITC-BSA was added for the final 15 min for endocytosis measurement. Endocytosis was decreased by PEG-SOD during normoxia and hypoxia, with significant difference only at 1 h for normoxia (P = 0.0065) and hypoxia (P = 0.036) by paired t-test.
hypoxia on endocytosis and NOS is compartmentalized. Clearly, NOS-3 is highly associated with the caveolar membrane in ovine fetal lung endothelial cells (50) and bovine lung MVECs (14), and our present functional data support such an association in ovine fetal and neonatal lung MVECs. Figure 5B demonstrates that caveolin-1 and NOS-specific antibodies separately increase endocytosis. Similarly, accumulation of NO and albumin from the same cells in the Transwell culture plates shows a parallelism (Fig. 6), and in other studies, activation of endocytosis with BSA increased NO production (Fig. 7), suggesting that caveolin-1 and NOS-3 are not just binding partners but may be, more importantly, a signaling couple with related functions. Activation of caveolin-1 appears to cause the dissociation of the two molecules, with an increase in their respective measurable activities (Fig. 1). Thus the integral protein caveolin-1 may serve a signaling role in endocytosis as well as in NOS function. However, because endocytosis can be upregulated by caveolin-1 antibody (51), our measurement of NO after activation of endocytosis is more likely due to caveolin-1 activation than general endocytosis activation. Cyclohextrin, which structurally modifies caveolae, enhanced NOS activity (Fig. 5, B and C). Therefore, the specific structural orientation of caveolin-1 in caveolae as it relates to NOS function as an inhibitor and how this is affected by various factors that alter endocytosis need further exploration. Caveolin-1 is a membrane protein forming a hairpin loop with both terminals in the cytosol. Inactive NOS is also a membrane-associated protein. From Fig. 5B, it appears that the accessibility of the two antibodies is not hindered by a membrane barrier and that the responses may be caveolar. Interestingly, in previous publications, a relation between caveolin-1 and NOS-3 protein expression, but not function, has been demonstrated by coimmunoprecipitation. NOS-3 is quantitatively immunoprecipitated by antibodies against caveolin-1 in endothelial cells, and, conversely, NOS-3 anti-serum immunoprecipitates caveolin-3 in cardiomyocytes (10). Furthermore, phosphorylated NOS-3 interacts with caveolin-1 during immunoprecipitation (13). These reports demonstrate a physicochemical relation between caveolin-1 and NOS-3, whereas our observations demonstrate a functional and a physical relation between caveolin-1 and NOS-3. We coimmunoprecipitated NOS protein (140 kDa) with caveolin-1 protein (22 kDa) from cell lysates of newborn MVECs subjected to 15 min of hypoxia (Fig. 6A). We also detected a ~160-kDa protein, which may represent NOS complexed with caveolin-1 in the cell lysates. Interestingly, crude cell lysate showed caveolin-1 bound to other proteins smaller than NOS, consistent with diverse signaling roles of caveolin-1. Caveolin-1 and NOS-3 were also colocalized in membrane regions of cell monolayers subjected to acute hypoxia (Fig. 6C).

PKC is highly localized to caveolae in lung (and other) endothelial cells (34) and has been shown outside the cell to interact with caveolin (41) and to affect the morphology and function of caveolae (53). Phorbol ester stimulation of PKCγ induces its interaction with caveolin-1 in lipid rafts, leading to morphological changes (31). We therefore used PMA to investigate the effect of PKC activation on endocytosis and NO production. Incubation of endothelial cells with 45 μM PMA for 24 h decreased endocytosis (20.35%, P < 0.001) and NO release (19.32%, P < 0.001) in normoxia (Fig. 10). The PKC interaction with NOS has been reported to be inhibitory (6, 28, 37), but results of interaction with caveolin or caveolae are varied (53, 55). Our data showing PKC inhibition of endocytosis are related to published reports that PKC isoforms and PKC activation cause flattening of caveolae and inhibition of endocytosis (53). The mechanism by which hypoxia decreases NOS activity, causing vasoconstriction, has been attributed to PKC (58), a tighter coupling of NOS-3 with caveolin (40), or a decrease in heat shock protein 90 (54). Hypoxia causes induction and phosphorylation of PKC (17), and our functional data show that hypoxia and PKC activation with PMA decrease NOS-3 and caveolin-1 functions. To further examine the role of PKC, we used synthetic caveolin peptide [which has the conserved consensus PKC phosphorylation site of caveolin (57)] plus caveolin-1/3 polyclonal antiserum against the same amino acid sequence 19–41 to prevent PKC phosphorylation of endogenous caveolin. This sequence is 77% homologous to caveolin-1 from mouse, human, and other species, although its homology with sheep caveolin-1 is unknown. Addition of the synthetic caveolin peptide raised FITC-BSA endocytosis by 16% in normoxia (P < 0.05) and by 25% in hypoxia (P < 0.001), suggesting that the peptide, similar to caveolin and caveolin peptides (44, 60), is recruited in some way in the functioning of caveolae. Alternatively, the peptide, as we expected, may have been substituted for endogenous caveolin for phosphorylation and inhibition by PKC, in which case PKC phosphorylation of caveolin-1 appears greater in hypoxia than in normoxia. Caveolin peptide also raised NO release in the cell-bathing medium in normoxia by 12.32% (P < 0.05). PKC activity in newborn MVECs shows a compartmental shift between normoxia and hypoxia: it is higher in the nuclear extracts in hypoxia and higher in the cytosolic extracts in normoxia (Fig. 10). Interestingly, the intense expression of phosphorylated threonine in the membranous region observed in hypoxia is not seen in normoxia. Instead, normoxic cells show phosphorylated threonine within organelles in the cytosol (Fig. 10).

Gαi is thought to transduce receptor activation to caveolar endocytosis (8, 39). We have shown that the Gαi inhibitor PTX increased FITC-BSA endocytosis in normoxia (14.89%) and hypoxia (21.13%), indicating that sustained Gαi activity is a negative, rather than a positive, regulator of endocytosis in these cells. Consistent with our hypothesis that activation of caveolin and its dissociation from NOS-3 lead to activation of NOS-3, PTX also increased NO production in normoxia (5.71%) and hypoxia (13.41%, P < 0.01). Caveolin has been shown to directly interact with the G protein α-subunit (30). From our data, we speculate that there could be more inhibitory Gαi-caveolin interaction in hypoxia than in normoxia. Endocytosis and NO production were enhanced more in hypoxia than in normoxia.

MVECs produce more ROS in hypoxia than in normoxia. Figure 12 indicates that intracellular ROS do modulate caveolar function after 1 h of incubation with PEG-SOD. However, from our other ongoing work, we realize that experimental conditions and cellular processes delicately determine the accumulation of specific ROS species and that the biological effect of ROS further depends on the site of accumulation of the species (e.g., intracaveolar, cytosolic, and juxtaaplasmaellmal). In Fig. 12, there was no difference at 15 min in endocytosis in MVECs in which intracellular scavenging of
superoxide/accumulation of hydrogen peroxide was increased. Thus we do not attribute the effects we observed in this study of caveolin-1-NOS-3 interaction in normoxia and hypoxia to the action of these ROS.

Overall, our data show that oxygen definitely modulates the functional relation between caveolin-1 and NOS-3 (Tables 1 and 2). Caveolin-1 and NOS-3 functions are decreased by hypoxia. In addition, it appears that unlabeled albumin, caveolin peptide, and PTX, which increase these functions, tend to have a greater effect in normoxia than in hypoxia. PMA and L-NNA, which decrease these functions, tend to have a greater effect in normoxia (Tables 1 and 2). Therefore, there is a link between oxygen signaling and intracellular modulators of NO production and caveolar function. Estrogen receptors are coupled to NOS-3 through Go, i.e., our data suggest that, similarly, an NOS-3-coupled cell surface G protein-coupled receptor is sensitive to oxygen in the lung cells and relays through Go and caveolin. Cell surface G protein-coupled receptors are modulated by atmospheric signals such as light and taste bud cell signals.

The relation between endocytosis and NO production is interesting. Kabbani and Cassin (26) speculated that lung liquid reduction and elevation of pulmonary blood flow at birth may be controlled by a common transduction pathway. Our data suggest that oxygen could activate fluid-phase endocytosis directly. Caveolae-mediated clearance is not considered a mechanism for physiological alveolar clearance at birth, which is attributed to other mechanisms (42). If it is important for perinatal alveolar clearance, further investigation may reveal unknown but significant etiology of neonatal pulmonary diseases.

We conclude that a change in PO2 from hypoxia to normoxia in fetal and neonatal pulmonary MVECs enhances caveolin-1 and NOS-3 functions (Figs. 1 and 13). Caveolin-1 and NOS-3 are physically linked, and activation of caveolin-1 causes their dissociation and enhancement of their respective activities. Hence, activation of caveolar endocytosis with albumin leads to increased NO production. It appears that the effect of PO2 is not directly on caveolin-1 or NOS-3 but that oxygen acts through inherent intermediate signaling involving at least Go and PKC as endogenous transducers (Fig. 13). The involvement of Go suggests that the original sensor of oxygen is a cell

| Table 2. Effect of modulators on NOS-3 and caveolin-1 activities |
|---|---|---|
| Modulator | Normoxia | Hypoxia |
| **NO** | | |
| L-NNA | ↓44.6 | ↓20 |
| Arginine depletion | ↓42.6 | ↓19.9 |
| L-NNA + arginine depletion | ↓81 |
| BSA | | |
| Cell lysates | ↑11.8 | ↑20 |
| Cell medium | ↑11 | ↑10.5 |
| Caveolin peptide | ↑12 |
| PMA | ↑19.3 | ↓11.8 |
| PTX | ↑14.9 | ↑21.1 |
| **Endocytosis** | | |
| PMA | ↓20.4 |
| PTX | ↑5.7 | ↑13.4 |
| Caveolin peptide | ↑16.3 | ↑25 |

Values are expressed as percentages. NOS-3, NO synthase-3; L-NNA, N-nitro-L-arginine; PTX, pertussis toxin. Downregulators tended to have a greater effect in normoxia and upregulators to have a greater effect in hypoxia.
surface G protein-coupled receptor. The lung MVECs form >25% of MVECs in the body. The signaling we observed may be peculiar to lung MVECs and further peculiar to the fetal/neonatal cells or may be found in other MVECs. Our speculation is that a PO2-sensitive molecular or genomic switch is relevant in the lung. The scheme in Fig. 1 proposes that two necessary birth processes in the lung, rapid alveolar clearance for oxygen diffusion and vasodilation for increased blood and oxygen distribution, could be initiated or enhanced by a common switch that is sensitive to PO2. In the perinatal period, the role of NO in vascular homeostasis makes the regulation of NO by caveolin-1 an important therapeutic target.

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