

# Hypoxia Modulates the Barrier and Coagulant Function of Cultured Bovine Endothelium

## Increased Monolayer Permeability and Induction of Procoagulant Properties

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### Abstract

Exposure of cultured endothelium to environments with low concentrations of oxygen, in the range of those observed in pathophysiologic hypoxemic states *in vivo*, compromises cellular barrier and coagulant function. An atmosphere with  $PO_2 \approx 14$  mm Hg was not lethally toxic to endothelial cultures, but cells became larger and exhibited small intercellular gaps. At low oxygen concentrations, passage of macromolecular tracers through hypoxic endothelial monolayers was accelerated in a time- and dose-dependent manner, presumably by a paracellular pathway via the gaps. Cell surface coagulant properties of the endothelium were also perturbed. At  $PO_2 \approx 14$  mm Hg thrombomodulin antigen and functional activity on the cell surface were diminished by 80–90%, and Northern blots demonstrated suppression of thrombomodulin mRNA. The decrease in thrombomodulin was twice as great compared with the general decline in total protein synthesis in hypoxia. In addition, expression of a direct Factor X activator developed under hypoxic conditions; the activator was membrane-associated and expressed on the surface of intact cultures, Ca-dependent, inhibited by  $HgCl_2$  but not PMSF, and had  $K_m \approx 25$   $\mu g/ml$  for the substrate at pH 7.4. Synthesis of the activator was blocked by inclusion of cycloheximide, but not warfarin, in the culture medium. These results demonstrate that endothelial function is perturbed in a selective manner in the presence of low concentrations of oxygen, providing insights into mechanisms which may contribute to vascular dysfunction in hypoxemic states. (*J. Clin. Invest.* 1990. 85:1090–1098.) ischemia • thrombosis • thrombomodulin • vascular injury

### Introduction

Hypoxemia is a frequent feature of disorders of the circulatory system, especially those associated with ischemic cardiovascular disease. The effects of low concentrations of oxygen on cellular functions are quite complex, and include changes in a spectrum of cellular properties ranging from redirection of protein synthesis with the expression of oxygen-regulated pro-

teins (1–4) to the production of  $\alpha_1$ -adrenergic receptors and mitogens/inhibitors for smooth muscle cell growth (5–8). Thus, concomitant with the well-known hypoxia-mediated enhancement of glycolysis and suppression of aerobic metabolism (9, 10), biosynthetic processes and changes in receptor expression are set into motion which can actively modulate cellular phenotype.

Of the many changes in homeostatic mechanisms in hypoxemia, two are of particular clinical importance: increased vascular permeability and a prothrombotic tendency (11–18). The clinical consequences of these perturbations of vascular function are evident in the pulmonary edema which results from the hypoxia of high altitude, and the cerebral edema which may occur in hypoxemia (11–15). Furthermore, formation of venous thrombi is closely associated with the development of severe local hypoxemia in venous circulation (16–18). Since endothelium has a major role in the regulation of barrier function and intravascular coagulation, we have examined the effects of hypoxemia on these cells which form the luminal vascular surface.

We have exposed cultured bovine aortic endothelium to low concentrations of oxygen and assessed permeability of the monolayer and cell surface coagulant properties. Our studies indicate that hypoxia increases endothelial permeability and shifts the balance of cell surface coagulant properties, producing a Factor X activator that promotes coagulation and suppressing the anticoagulant cofactor thrombomodulin. These observations suggest that hypoxic endothelium may contribute to the pathogenesis of vascular disorders in which intravascular oxygen concentrations are decreased.

### Methods

*Culture of endothelial cells and conditions for achieving hypoxia.* Bovine aortic endothelial cells were grown from aortas of newborn calves in minimal essential medium supplemented with penicillin-streptomycin (50 U/ml–5  $\mu g/ml$ ), Hepes (pH 7.4; 10 mM), glutamine and fetal calf serum (10%; HyClone Laboratories, Logan, UT.) as described (19, 20). Cultures were characterized as endothelial based on morphologic criteria, and immunofluorescence for protein S, von Willebrand factor, and thrombomodulin (21–23). Cells were separated for subculture with trypsin/EDTA. For experiments, cells from different aortas were grown to confluence in 9.6-cm<sup>2</sup> wells (Becton-Dickinson Labware, Lincoln Park, NJ; Lux Permanox, Miles Laboratories, Inc., Naperville, IL) and used from passages 3–16. Permeability studies employed cultures grown as described previously (24) on 6.5-mm diam polycarbonate membranes (pore size 0.4  $\mu m$ ), mounted on polystyrene inserts (Transwell plates, Costar Data Packaging Corp., Cambridge, MA), and used for studies 14 d after plating. Cultures were characterized based on cell density, determined using a Coulter counter (model ZM, Coulter Electronics, Luton, England), time in culture, and labeling index, using a kit from Amersham Corp. (Arlington Heights, IL). For experiments, endothelial cultures were grown to confluence (7–10 d after plating; labeling index < 1%;  $2 \times 10^5$  cells/cm<sup>2</sup>) in an ambient

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air atmosphere, and then transferred to the hypoxia chamber for further study.

Confluent endothelial cultures were made hypoxic by removing the growth medium and substituting it with fresh medium which had been equilibrated with a gas mixture containing the desired concentration of oxygen (the balance of the atmosphere was made up by 5% carbon dioxide and nitrogen). Before adding this medium to the cells, dissolved gas analysis was performed (model ABL-2, Radiometer, Copenhagen, Denmark). Cultures were maintained under hypoxia using either sealed glass desiccators (equilibrated with an atmosphere containing the desired gas concentrations) placed in a 37°C oven or an incubator attached to the hypoxia chamber (see below) which could maintain a humidified atmosphere with low oxygen concentrations (Coy Laboratory Products, Ann Arbor, MI; Forma Scientific, Marietta, OH). This apparatus established the hypoxic atmosphere by purging the chamber with a mixture containing the desired concentration of gases (O<sub>2</sub>, N<sub>2</sub>, CO<sub>2</sub>). The oxygen content was continuously monitored and regulated using an oxygen analyzer which controlled an automated valve system. At the indicated times, cultures were placed in the hypoxia chamber (Coy Laboratory Products), an airtight glove box, in which experiments were carried to completion without exposing endothelium to ambient air. At intervals throughout these experiments, the oxygen content of culture medium bathing the cells was repeatedly analyzed. Values shown in the figures are the oxygen pressure of the medium. During the course of these experiments, pH of the medium remained constant. In certain experiments, either cycloheximide (0.1 µg/ml for 48 h; Sigma Chemical Co., St. Louis, MO) or the warfarin derivative 3(α-acetonyl benzyl)-4-hydroxycoumarin (1 µg/ml; Sigma Chemical Co.) was added to the growth medium during the incubation period under hypoxic conditions.

The protocols used included exposure to several levels of hypoxia (PO<sub>2</sub>'s of 12, 19, 47, 147 mm Hg) for variable periods of time (12, 24, 48, 72, 82, 96, 120 h).

Release of lactate dehydrogenase into the medium by endothelial cells exposed to hypoxia was evaluated using a commercially available kit (Sigma Chemical Co.).

Endothelial monolayers were incubated with purified recombinant human tumor necrosis factor/cachectin-α (≈ 10<sup>8</sup> U/mg; generously provided by Dr. P. Lomedico, Hoffmann-LaRoche, Inc., Nutley, NJ) and tissue factor activity was studied as described below.

**Determination of endothelial monolayer permeability.** Confluent endothelial monolayers plated on Transwell inserts (see above) were exposed to hypoxia, washed with Earle's balanced salt solution, and then minimal essential medium containing fetal calf serum (5%) was added (all solutions were equilibrated with the gas mixture being used for that experiment and were carried out in the hypoxia chamber). Medium was added to both the inner and outer (corresponding to upper and lower) chambers so that the oncotic and hydrostatic pressures in the two chambers remained equal. A radiolabeled marker was then added at trace concentrations, either [<sup>3</sup>H]inulin (3 µg/ml, 271 mCi/g; New England Nuclear, Boston, MA), [<sup>3</sup>H]sorbitol (38 ng/ml, 24 Ci/mmol; New England Nuclear), or <sup>125</sup>I-albumin (150 ng/ml, 5,000 cpm/ng), to the upper chamber. Radiolabeled albumin was prepared by the lactoperoxidase method (25) using Enzymobeads (Bio-Rad Laboratories, Sacramento, CA), desalted on a Sephadex G25 column (Pharmacia, Inc., Piscataway, NJ) and dialyzed exhaustively against Hanks' balanced salt solution. The final product used for permeability studies was > 95% precipitable in 10% trichloroacetic acid. The volume of fluid in the chambers was not changed significantly (< 5%) as a result of sampling of wells during the experiment. Transport of tracers from the inner to outer chamber, i.e., across the endothelial monolayer, was determined by dividing radioactivity emerging in the outer well by radioactivity in remaining in the inner well (measured from duplicate 0.005-ml samples from each well).

**Assessment of endothelial coagulant properties.** Endothelial thrombomodulin activity and antigen were assessed after exposing cultures to hypoxia, as described previously (23, 26). In brief, functional assays for cell surface thrombomodulin were performed by

washing cultures four times with Hanks' balanced salt solution, and then incubating for 60 min at 37°C in 10 mM Hepes, pH 7.45, 137 mM NaCl, 11 mM glucose, 4 mM KCl, 2 mM CaCl<sub>2</sub>, and 2 mg/ml bovine serum albumin containing protein C (100 µg/ml) and thrombin (0.1 U/ml). The assay was carried out in the hypoxia chamber in the same atmosphere to which the cultures had been previously exposed and all buffers were preequilibrated with this gas mixture. Formation of activated protein C was terminated by the addition of antithrombin III (100 µg/ml) and the amount of enzyme formed was determined using a chromogenic assay, hydrolysis of the substrate Lys-Pro-Arg-*p*-nitroanilide (Spectrozyme, American Diagnostica, NY). Enzyme concentration was determined by comparison with a standard curve made in the presence of known amounts of activated protein C. This assay has been described previously (26). Protein C, antithrombin III (inhibitory activity of 1 U thrombin per µg), and thrombin (2,500 U/mg) were prepared by standard procedures (27–29). Each of these purified proteins was homogeneous on SDS-PAGE (30).

Total thrombomodulin antigen was assessed by radioimmunoassay of endothelial cell detergent lysates (1% NP-40) prepared in the presence of protease inhibitors, phenylmethylsulfonyl fluoride (PMSF, 2 mM; Sigma Chemical Co.) and leupeptin (0.3 mM; Boehringer Mannheim Diagnostics, Inc., Houston, TX). The radioimmunoassay was carried out by a modification of the procedure described for human Factor IX (31), which is similar to that of Moore et al. (32). In brief, the assay was performed in 1.5-ml microcentrifuge tubes by adding <sup>125</sup>I-bovine thrombomodulin (5 µl), antithrombomodulin antiserum (10 µl), and incubation buffer (20 µl; Tris, 20 mM, pH 7.4, NaCl, 0.1 M, and NP-40, 1%). Selected tubes contained either samples or thrombomodulin standard diluted in the assay buffer (20 µl). Tubes were incubated overnight at 4°C, after which 50 µl of a 10% suspension of *Staphylococcus* protein A (IgGSorb, Enzyme Center, Inc., Walden, MA) was added to each tube for 30 min at 21°C. Incubation buffer (500 µl) was added next, and the tubes were centrifuged for 3 min at 13,000 rpm. The supernatant was aspirated, and the bottom of each tube containing the pellet was cut off with a hot wire and counted. Bovine thrombomodulin was purified from lungs by the method of Jacobowski et al. (33) and radiolabeling of preparations was carried out by the lactoperoxidase procedure (25), using Enzymobeads. Antibody to thrombomodulin was prepared in rabbits by standard methods (34). The sensitivity of the thrombomodulin radioimmunoassay was 10 ng/ml, which corresponded to 80% binding on the standard curve. Using this assay, the thrombomodulin content of untreated endothelial cultures was ~ 40–60 ng/10<sup>6</sup> cells.

Levels of thrombomodulin antigen were compared with total protein synthesis and fibronectin production. The effect of hypoxia on overall protein biosynthesis was assessed by evaluating cellular incorporation of [<sup>3</sup>H]leucine (60 Ci/mmol; New England Nuclear) into trichloroacetic acid precipitable material using the method described by Madri et al. (35). Briefly, [<sup>3</sup>H]leucine (20 µCi) was added to hypoxic cultures 12 h before harvesting the cells. 12 h later, the supernatant and cells were precipitated in ice cold trichloroacetic acid (10%). After 24 h at 4°C, the precipitates were collected and washed by filtration over Whatman No. 1 filter paper (Whatman Inc., Clifton, NJ). The papers were placed in vials, scintillation cocktail was added (Aquassure, New England Nuclear) and the mixtures were counted in a RackBeta counter (LKB Instruments, Inc., Gaithersburg, MD). Endothelial elaboration of fibronectin was assessed by obtaining aliquots of culture supernatant, and determining their fibronectin content by radioimmunoassay. The radioimmunoassay for fibronectin was carried out by the same procedure described above for thrombomodulin except that the tracer was <sup>125</sup>I-bovine fibronectin, the antibody was a polyclonal anti-bovine fibronectin (Calbiochem-Behring Corp., San Diego, CA), and the incubation of first antibody with the assay mixture was for 8 h at 25°C. Purified bovine fibronectin, also obtained from Calbiochem-Behring Corp., was radiolabeled by the lactoperoxidase method as described above for albumin. The sensitivity of the fibronectin radioimmunoassay was 0.2 µg/ml, which corresponded to 80% binding on the standard curve.

Northern blots to assess levels of thrombomodulin and fibronectin mRNA were carried out by extracting total RNA from cells using the guanidinium thiocyanate procedure (36), electrophoretic fractionation of the RNA on a 1.2% agarose gel (37), and transfer to nitrocellulose. cDNA probes for thrombomodulin (generously provided by Dr. E. Sadler, Wash University, St. Louis, MO) (38) and fibronectin (generously provided by Dr. R. Hynes, Massachusetts Institute of Technology, Cambridge, MA) (39) were labeled using random hexamer labeling (random primed DNA labeling kit, Boehringer Mannheim Biochemicals, Indianapolis, IN). Hybridization of cDNA probes to normal and hypoxic RNA was performed at 42°C as described previously (40).

Study of endothelial cell procoagulant properties was carried out using coagulant and amidolytic assays. The coagulant assay was performed on either intact monolayers (9.6 cm<sup>2</sup>) or cells scraped with a rubber policeman to obtain a suspension ( $\approx 10^6$  cells/ml). The cells in suspension were > 90% viable, based on trypan blue exclusion. The cells were washed three times with veronal buffer (once with veronal buffer containing 5 mM EDTA over 3 min and twice with veronal buffer without EDTA) and then 0.2 ml of the same buffer was added along with citrated bovine plasma (0.2 ml) and CaCl<sub>2</sub> (20 mM, 0.2 ml), and the mixture was incubated at 37°C for the first visual evidence of a fibrin clot. The Factor Xa clotting assay was performed by incubating purified Factor X (50 µg/ml) with hypoxic or normoxic endothelial cells for the indicated times, removing aliquots (60 µl) and adding them to Factor VII/X-deficient bovine plasma (60 µl, Sigma Chemical Co.) along with cephalin (60 µl) and CaCl<sub>2</sub> (20 mM, 60 µl). Enzyme concentration was determined by comparison with a standard curve made with known amounts of Factor Xa. The amidolytic assay for Factor Xa (41) was performed by incubating endothelium with Factor X (50 µg/ml) and testing aliquots of reaction medium for Factor Xa activity with the chromogenic substrate methoxycarbonyl-D-cyclohexyl-Gly-Gly-Arg-p-nitroanilide (Spectrozyme FXa, American Diagnostica). Change in optical density at 405 nm was measured and compared with a standard curve generated with purified Factor Xa. This assay was sensitive to 0.4–0.5 nM Factor Xa. Where indicated, after the initial exposure to hypoxia, endothelial cells were further incubated in the same hypoxic environment in the presence of either antibody to tissue factor (10 µg/ml, 1 h at 37°C) (blocking anti-bovine tissue factor monoclonal antibody was generously provided by Dr. R. Bach, Mt. Sinai School of Medicine, New York) (42), mercury chloride (0.1 mM, 30 min at 37°C), or PMSF (1 mM, 30 min at room temperature) before carrying out the coagulant assay. Cultures were then washed extensively and the Factor X activation assay was performed. Activation of Factor X by hypoxic endothelium was also studied by examining cleavage of <sup>125</sup>I-Factor X on SDS-PAGE. Endothelial cultures, normoxic or hypoxic, were incubated with radiolabeled Factor X and samples were prepared for reduced SDS-PAGE (30).

For these studies Factors X (100 U/mg), IX (220 U/mg), and prothrombin (13 U/mg) were purified to homogeneity by previously described methods (27, 43, 44). Purified Factor X had no detectable Factor VII activity: prolonged incubation with tissue factor did not lead to Factor Xa formation. Factor X was radiolabeled by the lactoperoxidase method using Enzymobeads as described previously for albumin. <sup>125</sup>I-Factor X was > 95% precipitable in 10% trichloroacetic acid and had a specific radioactivity of 5,000 cpm/ng. Factor X and radiolabeled Factor X were activated by incubation with Russell's viper venom (ratio of 1:100 for enzyme to substrate, generously supplied in purified form by Dr. Richard Hart, American Diagnostica). Monospecific antibodies to bovine Factors VII and IX were provided by Dr. W. Kiesel (University of New Mexico School of Medicine, Albuquerque, NM) and antibody to bovine Factor VIII was provided by Dr. G. Vehar (Genentech, South San Francisco, CA).

In one experiment, the procoagulant activity of tumor necrosis factor/cachectin-treated endothelium was examined using a two-stage plasma recalcification clotting assay, as described previously (26, 45). Inhibition of endothelial tissue factor induced by tumor necrosis factor (26, 46) was studied with purified antibody to tissue factor (10 µg/ml).

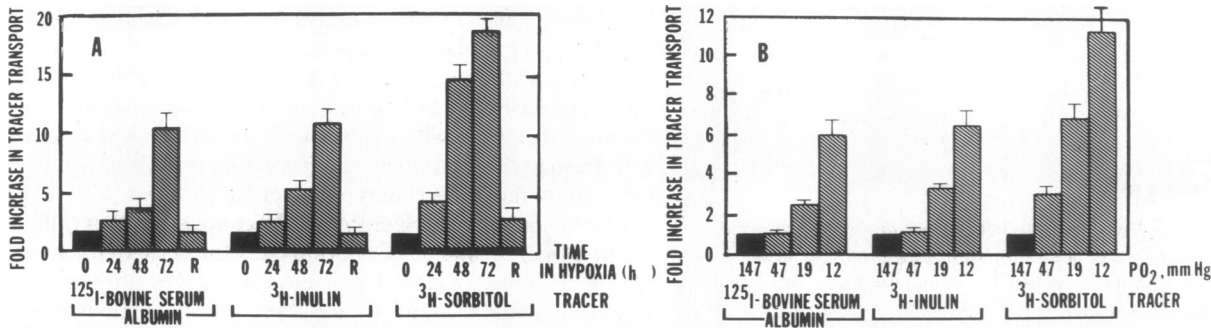
To examine modulation of vessel wall coagulant properties using fresh native endothelium, an experiment was carried out with aortic segments obtained from calves immediately after sacrifice (Max Cohen, Inc., New York). The vessel segments were incubated for 24 h in complete endothelial growth medium and exposed either to a normoxic or hypoxic (PO<sub>2</sub>  $\approx$  14 mm Hg) humidified environment containing 5% CO<sub>2</sub>. Then, the segments were washed with Hanks' balanced salt solution (first with and then without 5 mM EDTA), placed in a template device, and functional assays for thrombomodulin and the Factor X activator were carried out as described above for cultured cells. Hypoxic vessel segments were maintained in the hypoxic atmosphere during the entire assay period. The template device consisted of two sheets of lucite, a top sheet with regularly spaced holes (surface area  $\approx$  1.5 cm<sup>2</sup>) and a solid bottom sheet. Vessel segments were placed between the two sheets of the template such that watertight wells with exposed native endothelium were formed. This system has been described by us previously (47).

## Results

*General properties and barrier function of hypoxic endothelial cultures.* Endothelial cell cultures tolerated the hypoxic environment (as low as PO<sub>2</sub>  $\approx$  14 mm Hg) well with no evidence of cell death, based on trypan blue exclusion and lactate dehydrogenase release, up to the longest incubation times examined, 4–5 d. This is consistent with previous observations (10). However, monolayers maintained in hypoxic conditions did exhibit alterations in the actin-based cytoskeleton and cell shape. Cells exposed to hypoxia (PO<sub>2</sub>  $\approx$  14 mm Hg) for 24 h appeared flatter, their peripheral bands of circumferential stress fibers appeared diffuse, and in the central cytoplasm reticular networks of thin actin filaments developed, compared with normoxic controls. These changes became more accentuated by 48 h. Concomitant with alterations in the actin-based cytoskeleton, multiple small gaps developed between apposing cells evident by scanning electron microscopy. Each of these changes was reversible, and within 48 h of return to an ambient air atmosphere cultures closely resembled their normoxic counterparts.

The presence of intercellular gaps in the monolayer suggested that barrier function would be perturbed. Confluent endothelial monolayers grown on microporous polycarbonate membranes form a barrier restricting the passage of macromolecules and lower molecular weight solutes (Fig. 1, A and B) (24, 48–50). Using this experimental system, cultures shifted to hypoxia (PO<sub>2</sub>  $\approx$  14 mm Hg) demonstrated an increase in the passage of macromolecular tracers across the monolayer. The hypoxia-induced diffusional defect was dependent on both the amount of time cultures were incubated under hypoxic conditions (Fig. 1 A) and the degree of hypoxia (Fig. 1 B). Increased permeability of the endothelial monolayer to [<sup>3</sup>H]-sorbitol was evident within 24 h and a similar increase in the passage of [<sup>3</sup>H]inulin and <sup>125</sup>I-albumin was seen within 48 h. After 72 h under hypoxic conditions, cultures showed a maximal response, with respect to increased permeability, which was not changed during an additional 24 h in this environment (data not shown). Restitution to an ambient air environment led to a reversal of these alterations in monolayer permeability within 48 h (Fig. 1 A) concomitant with restoration of normal morphology of the monolayer.

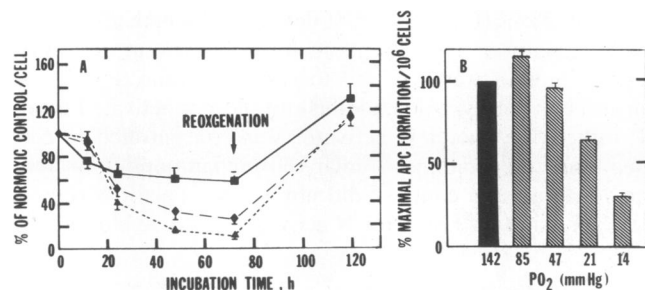
*Hypoxia-mediated suppression of endothelial thrombomodulin.* Because in the quiescent state anticoagulant activities predominate on the vessel surface, the integrity of a central



**Figure 1.** Permeability of endothelial monolayers exposed to hypoxia: effect of incubation time and oxygen concentration. (A) Dependence of permeability on incubation time in hypoxia. Confluent endothelial monolayers grown on filters were incubated in an environment with normal oxygen content (solid bars) or with  $P_{O_2} \approx 14$  mm Hg (cross-hatched bars), and permeability assays were carried out at the indicated times (24, 48, 72 h) by adding tracers ( $^{125}\text{I}$ -albumin,  $^3\text{H}$ -inulin,  $^3\text{H}$ -sorbitol) to the compartment above the monolayer. R cultures placed in an ambient air atmosphere (recovery) for 48 h after 72 h in hypoxia. (B) Dependence of permeability on oxygen concentration. Monolayers on filters were incubated for 48 h in an atmosphere containing the indicated concentration of oxygen, and then permeability assays were carried out by adding tracers ( $^{125}\text{I}$ -albumin,  $^3\text{H}$ -inulin,  $^3\text{H}$ -sorbitol) to the compartment above the monolayer. In each case (A and B), transfer of tracer to the compartment below the monolayer was assessed after a 4-h incubation period of endothelium with the tracers as described in the text, and is shown as the ratio of transfer of tracer in hypoxic cultures to transfer of tracer in normoxic cultures (fold increase in hypoxia compared with normoxic controls). Transfer across normoxic cultures for each tracer in 4 h was arbitrarily defined as 1. The mean  $\pm$  SD is shown.

anticoagulant mechanism closely linked to the endothelium, the protein C/protein S pathway (23), was examined on hypoxic endothelial cultures by measuring thrombomodulin, a cell surface anticoagulant cofactor that facilitates thrombin-mediated activation of protein C (23).

Exposure of endothelium to an atmosphere with  $P_{O_2} \approx 14$  mm Hg led to a fall in cell surface thrombomodulin activity and thrombomodulin antigen (Fig. 2 A). Decreased thrombo-



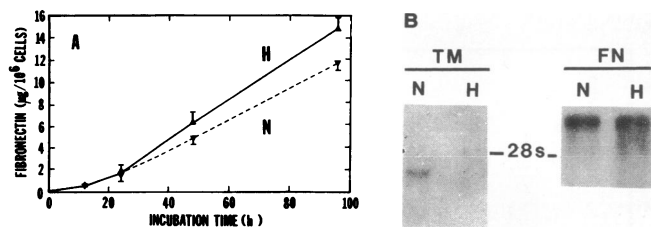
**Figure 2.** Hypoxia-induced suppression of endothelial thrombomodulin. (A) Effect of incubation time. Confluent endothelial monolayers were incubated in an atmosphere with  $P_{O_2} \approx 14$  mm Hg and thrombomodulin function ( $\blacklozenge$ )/antigen ( $\blacktriangle$ ) was assessed at the indicated times. Functional assays of endothelial-dependent activated protein C formation were carried out on intact monolayers in an hypoxic environment by incubating cultures with thrombin and protein C. For the radioimmunoassay, samples were obtained by solubilizing cultures in detergent-containing buffer with protease inhibitors. Total protein synthesis ( $\blacksquare$ ) was estimated by determining the extent of incorporation of  $^3\text{H}$ -leucine (added in each case 12 h before samples were harvested) into material precipitable in trichloroacetic acid. Details of methods are described in the text. Experimental observations in hypoxic cultures are shown as a percentage of the value in normoxic cultures per cell. (B) Effect of oxygen concentration. Confluent endothelial monolayers were incubated for 48 h in an atmosphere with the indicated oxygen concentration and thrombomodulin functional activity was assessed as described in the text. APC, activated protein C. Data are shown as percent APC formation in normoxic controls/cell, the latter arbitrarily designated as maximal APC formation, 100%. The mean  $\pm$  SD is shown.

modulin was observed within 24 h of exposure to hypoxia and reached a maximum by 72 h, at which time cell surface thrombomodulin activity and total cellular thrombomodulin antigen had declined by  $\sim 80\%$ . Hypoxia-mediated suppression of thrombomodulin was also dependent on the oxygen concentration, with a significant fall in thrombomodulin activity occurring at lower levels of oxygen (Fig. 2 B).

To examine mechanisms by which hypoxia suppressed thrombomodulin, first the effect of hypoxia on total protein synthesis was studied. Based on the incorporation of  $^3\text{H}$ -leucine into material precipitable in trichloroacetic acid, endothelial protein synthesis fell within 12 hr of exposure to hypoxia (Fig. 2 A), and reached its lowest levels after 24–48 h (a decline of  $\sim 40\%$  compared with normoxic cultures). In contrast, although thrombomodulin initially fell more slowly, by 48 h the decline was much greater. These results suggest that there might be a selective suppression of thrombomodulin in hypoxia. This interpretation was further supported by experiments demonstrating that another, unrelated macromolecular product of endothelium, fibronectin, was produced in normal or even slightly increased amounts in hypoxia (Fig. 3 A). Finally, Northern blots of endothelial RNA hybridized with cDNA probes for thrombomodulin and fibronectin showed a marked decrease in thrombomodulin, but not fibronectin mRNA (Fig. 3 B).

Suppression of thrombomodulin in hypoxic endothelial cells was reversible: levels of thrombomodulin activity/antigen approaching (even exceeding) that of normoxic controls were observed within 48 h of restitution of an ambient air atmosphere (Fig. 2 A).

**Hypoxia-mediated induction of endothelial procoagulant activity: induction of a Factor X activator.** Exposure of endothelial cultures to hypoxic conditions induced clot-promoting activity, assessed by a shortening of the recalcification time of plasma (Fig. 4). Expression of cell surface procoagulant activity was dependent on the time cultures were maintained in hypoxic conditions, being evident by 24 h and maximal by 72 h (Fig. 4 A). Similar activity was not observed with intact or frozen-thawed endothelium grown in ambient air. Induction



**Figure 3.** Effect of hypoxia on endothelial production of fibronectin and thrombomodulin. (A) Elaboration of fibronectin by hypoxic endothelial cultures. Confluent endothelial monolayers were incubated in an ambient air (N, dashed line) or hypoxic (H, solid line) environment, and elaboration of fibronectin into the medium was determined by radioimmunoassay as described in the text. Data shown represent the mean  $\pm$  SD. (B) Northern blot of hypoxic endothelial cell RNA hybridized with cDNA probes for thrombomodulin and fibronectin. Confluent endothelial cultures were incubated in an ambient air (N) or hypoxic (H) environment for 18 h and then RNA was extracted. Samples containing  $\approx 10 \mu\text{g}$  of total RNA were applied to each lane of the gel, transferred to nitrocellulose, and then hybridized with a cDNA probe to either thrombomodulin (TM) or fibronectin (FN). Details of methods are described in the text. The markers correspond to 28S. The thrombomodulin message is between 3.7 and 3.8 kb and the fibronectin message is  $\approx 8.2$  kb.

of clot-promoting activity was also dependent on the oxygen concentration, especially at lower oxygen tensions (Fig. 4 B).

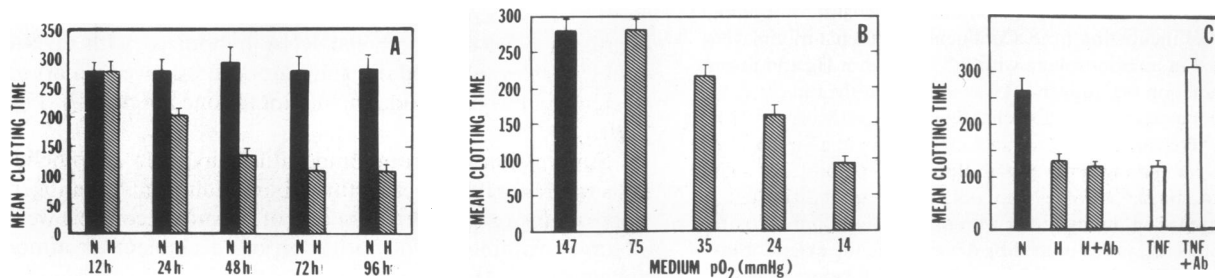
The results of previous studies demonstrating that exposure of cultured endothelial cells to cytokines and endotoxin induces tissue factor (26, 45, 46, 51) led us to examine whether hypoxic endothelium expressed tissue factor procoagulant activity. A blocking monoclonal antibody to bovine tissue factor which, under the same conditions, could reverse the procoagulant activity of tumor necrosis factor-treated endothelial cells (Fig. 4 C), failed to affect the clotting time of hypoxic endothelium (Fig. 4 C). These results suggested that a procoagulant activity other than tissue factor was responsible for the shortened clotting time observed with hypoxic endothelium. The possibility that hypoxic endothelium was directly activating Factor IX or prothrombin was tested by adding these purified coagulation proteins to hypoxic endothelium. However, no

activation was evident (based on coagulant, and, for thrombin, amidolytic assays) and no cleavage was observed on SDS-PAGE using radiolabeled tracers.

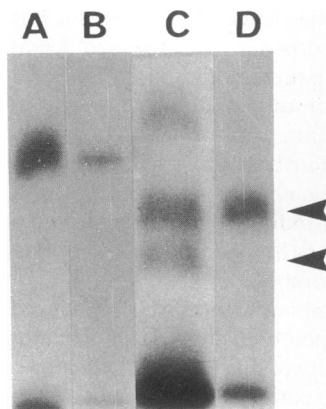
Studies with purified Factor X showed direct activation of the zymogen by intact hypoxic endothelial monolayers:  $^{125}\text{I}$ -Factor X incubated with hypoxic endothelial cells was cleaved with formation of products corresponding to  $M_r$ 's previously reported for the heavy chains of Factor X $\alpha$  and X $\beta$  (52) (Fig. 5, lane C) on reduced SDS-PAGE. In contrast, neither buffer controls (Fig. 5, lane A) nor normoxic cultures cleaved Factor X (Fig. 5, lane B). Control activation mixtures with Russell's viper venom and the same Factor X preparation demonstrated cleavage of Factor X with a predominance of the Factor X $\alpha$  heavy chain, as has been reported previously (53) (Fig. 5, lane D).

Further studies using a Factor Xa coagulant assay indicated that induction of endothelial-dependent activation of Factor X by hypoxia varied as a function of the incubation time in hypoxia and the oxygen concentration (Fig. 6, A and B). These results closely parallel the data concerning the clotting time of hypoxic cultures (Fig. 4, A and B). Similar results were obtained when Factor Xa formation was assessed using the synthetic substrate assay. Using both coagulant and amidolytic assays, activation of Factor X by hypoxic endothelium did not appear to be due to either the classical extrinsic or intrinsic systems: neither antibody to tissue factor, Factor VII, Factor IX, nor Factor VIII had any effect on Factor X activation (data not shown). In addition, Factor X activation by hypoxic endothelium was studied after washing cultures with EDTA-containing buffer under conditions which completely remove cell-associated Factor IX/IXa (47).

To characterize better the nature of the endothelial procoagulant activity resulting in Factor Xa formation under hypoxic conditions (Fig. 7), we first determined whether the Factor X activator of hypoxic endothelium was present in culture supernatants or was localized to cell membrane (Fig. 7 A): supernatants of hypoxic endothelium did not activate Factor X, rather, the Factor X activator was concentrated in cell membrane preparations. Similar cell preparations from normoxic cultures, in contrast, did not activate Factor X (Fig. 7 A). Expression of the Factor X activator could be blocked by



**Figure 4.** Hypoxia-induced expression of procoagulant activity by endothelium. Mean clotting time given in seconds. (A) Incubation time. Confluent endothelial monolayers were grown to confluence and then transferred to a hypoxic (H) atmosphere ( $\text{PO}_2 \approx 14$  mm Hg) for the indicated times given in hours. (B) Oxygen concentration. Confluent endothelial monolayers were grown to confluence and then transferred to an atmosphere with the indicated oxygen concentration for 48 h. In A and B procoagulant activity was assessed based on shortening of the clotting time of recalcified plasma as described in the text. N denotes normoxic controls. (C) Effect of blocking tissue factor monoclonal antibody on the procoagulant activity of hypoxic and tumor necrosis factor (TNF)-treated endothelial cultures. Confluent endothelial cultures were exposed to hypoxia ( $\text{PO}_2 \approx 14$  mm Hg) for 72 h and then their ability to shorten the clotting time was tested after preincubation for 30 min in the presence of anti-tissue factor antibody (H + Ab) or nonimmune mouse IgG (H). Control cultures were maintained in normoxia throughout the experiment (N). Details of experimental procedures are described in the text. Data shown are the mean  $\pm$  SD.



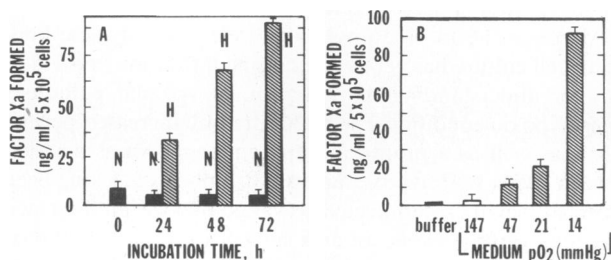
**Figure 5.** Factor X activation by hypoxic endothelium: reduced SDS-PAGE of reaction mixtures containing  $^{125}\text{I}$ -Factor X. Confluent endothelial monolayers were incubated for 72 h in hypoxia ( $\text{PO}_2 \approx 14$  mm Hg), washed in EDTA-containing buffer, and then incubated with  $^{125}\text{I}$ -Factor X ( $1 \mu\text{g}/\text{ml}$ ) for 1 h at  $37^\circ\text{C}$ . Other reaction mixtures contained either endothelial cells exposed only to normoxia, buffer, or Russell's viper venom in place of hypoxic endothelium. Aliquots of the reaction mixture

were analysed by SDS-reduced PAGE (12.5%) and autoradiography. Details of experimental methods are described in the text. Lanes A,  $^{125}\text{I}$ -Factor X incubated in buffer alone; lane B,  $^{125}\text{I}$ -Factor X incubated with normoxic endothelium; lane C,  $^{125}\text{I}$ -Factor X incubated with hypoxic endothelium; lane D,  $^{125}\text{I}$ -Factor X incubated with Russell's viper venom. Arrows correspond to the heavy chains of Factor X $\alpha$  ( $M_r \approx 27,000$  D) and Factor X $\beta$  ( $M_r \approx 23,000$  D). Molecular mass was determined from semilogarithmic plots based on the migration of standard proteins which were run on SDS-PAGE simultaneously.

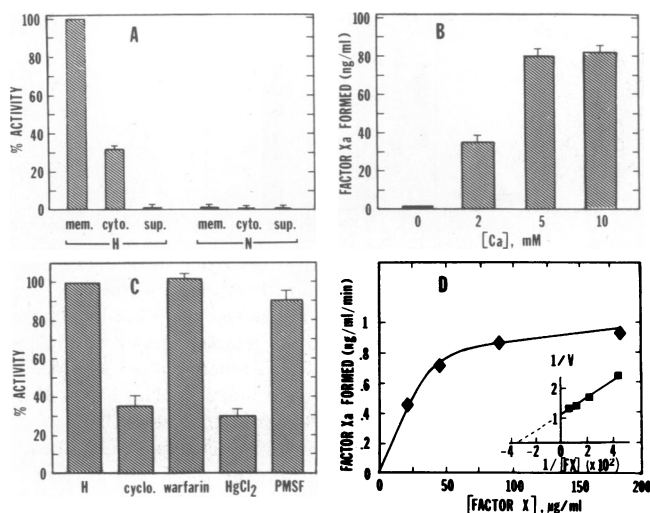
addition of cycloheximide to cultures at the time they were placed in hypoxia, although the warfarin derivative (which blocks release of protein S) (21) was without effect.

Once the Factor X activator was expressed, addition of the serine protease inhibitor phenylmethylsulphonyl fluoride had no effect on Factor X activation, but mercury chloride, an inhibitor of cysteine proteases, was an effective inhibitor. Activation of Factor X by hypoxic endothelial cultures occurred in a calcium-dependent manner with an apparent  $K_m$  of  $\approx 25 \mu\text{M}$  (Fig. 7, C and D).

These observations of direct Factor X activation by hypoxic cultured endothelial cells led us to inquire whether native

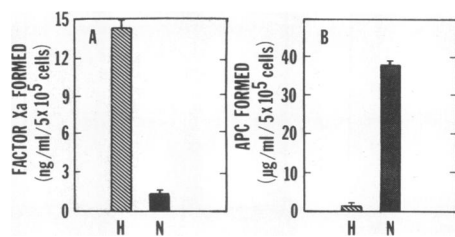


**Figure 6.** Factor X activation by hypoxic endothelium. (A) Effect of incubation time on the capacity of hypoxic endothelium to activate Factor X. Confluent endothelial monolayers were exposed to hypoxia ( $\text{PO}_2 \approx 14$  mm Hg) for the indicated time and washed with EDTA-containing buffer, and then their ability to directly activate purified Factor X was assessed as described in the text. (B) Effect of oxygen concentration on the capacity of hypoxic endothelium to activate Factor X. Confluent endothelial monolayers were incubated for 72 h in an atmosphere with the indicated oxygen concentration and washed with EDTA-containing buffer, and then their ability to directly activate purified Factor X was assessed. The bar labeled *buffer* refers to a reaction mixture containing Factor X incubated for 1 h with buffer alone in the absence of endothelial cells. In each case the mean  $\pm$  SD is shown.



**Figure 7.** Characteristics of the Factor X activator of hypoxic endothelial cells. (A) Association of the Factor X activator of hypoxic endothelium with cell membranes. Confluent monolayers of endothelial cells were exposed to hypoxia ( $\text{PO}_2 \approx 14$  mm Hg) for 48 h (under serum-free conditions), the supernatant was harvested, and the cells were scraped from the growth surface. After three freeze-thaw cycles, the suspension was subjected to ultracentrifugation ( $100,000 \text{ g}$  for 60 min) to pellet the membranes. Aliquots of culture supernatant, membranes, and lysates were then tested for their ability to activate Factor X in a coagulant assay. Samples were reconstituted to preserve the original relationship of  $\approx 10^6$  cells per 1 ml of culture supernatant. The identical experiment was also carried out using normoxic endothelial cultures. Details of methods are described in the text. (B) Effect of inhibitors in the Factor X activator of hypoxic endothelial cells. Confluent endothelial cultures were incubated in hypoxia ( $\text{PO}_2 \approx 14$  mm Hg) alone for 48 h (H, defined as 100%) or in the presence of cycloheximide (*cyclo*,  $0.1 \mu\text{g}/\text{ml}$ ) or the warfarin derivative 3( $\alpha$ -acetyl benzyl)-4-hydroxycoumarin ( $1 \mu\text{g}/\text{ml}$ ; warfarin). Samples were then assayed for their ability to activate Factor X using a coagulant assay as described in the text. In other experiments, all cultures were incubated for 72 h in hypoxia ( $\text{PO}_2 \approx 14$  mm Hg) and either  $\text{HgCl}_2$  ( $0.1 \text{ mM}$ ) or phenylmethylsulphonyl fluoride (*PMSF*,  $2 \text{ mM}$ ) was incubated with endothelial cells for 30 min to inactivate sensitive proteases. Cultures were then washed extensively with buffer and the coagulant Factor X activation assay was carried out. The percentage (mean  $\pm$  SD) of Factor X activation observed in cultures incubated in hypoxia alone is shown. (C) Effect of calcium concentration in the medium on activation of Factor X by hypoxic endothelium. Confluent endothelial cultures were incubated for 72 h in hypoxia ( $\text{PO}_2 \approx 14$  mm Hg), monolayers were then washed, and buffer with the indicated added calcium concentration was incubated with the cultures and Factor X activation was studied by the coagulant assay. (D) Effect of substrate concentration on the activation of Factor X by hypoxic endothelium. Endothelial monolayers were incubated in hypoxia ( $\text{PO}_2 \approx 14$  mm Hg) for 72 h and then Factor X activation in the presence of the indicated concentration of Factor X was studied by the coagulant assay. (Inset) Double reciprocal plot of the data in D calculated using nonlinear regression analysis applied to the Michaelis-Menten equation.

endothelium in situ, exposed to low oxygen concentrations, would behave similarly. To test this, calf aortic segments were exposed to hypoxia or an ambient air environment for their ability to activate Factor X (Fig. 8). Only the hypoxic organ cultures promoted Factor X activation, consistent with the results of the tissue culture experiments. In addition, hypoxic vessel segments showed reduced thrombin-mediated forma-



**Figure 8.** Activation of Factor X by hypoxic native aortic endothelium. Bovine aortae were obtained immediately after sacrifice, washed, and then incubated for 24 h in a normoxic (N) or hypoxic (H;  $PO_2 \approx 14$  mm Hg) environment. The template device was then applied to aortic segments, the wells were washed, buffer was added and the functional thrombomodulin assay and coagulant Factor X activation assay were carried out. APC, activated protein C. The mean  $\pm$  SD is shown. Details of experimental methods are described in the text.

tion of activated protein C, presumably reflecting decreased levels of functional thrombomodulin.

## Discussion

These studies demonstrate that hypoxia can perturb two endothelial functions, regulation of permeability and coagulation. In our experiments, changes in permeability of the endothelial monolayer were evident by 24–48 h of hypoxia (progressing up to 72 h), and were correlated with formation of intercellular gaps, which could form a paracellular pathway for fluid and solute escape. The extent of increased permeability observed in response to hypoxia is at least comparable to that observed in previous studies with agents such as tumor necrosis factor/cachectin and histamine using a similar experimental system, both of which affect vascular barrier function *in vitro* and *in vivo* (24, 55–59). Although endothelial monolayers which form on the artificial membrane surface used in these and other studies (24, 48–50) are more permeable than endothelium *in vivo*, they provide a useful model to examine the response to hypoxia and show clear differences with normoxic controls. Taken together, these observations suggest that our studies concerning the effect of an atmosphere with low oxygen content on permeability of monolayers of cultured aortic endothelium may provide insight into one of the mechanisms mediating increased leakage of protein and solute in hypoxia/hypoxemia. Rapid ascent to high altitude can be associated with the development of pulmonary edema, which is thought, at least in part, to be due to increased vascular permeability (11–14). And a recent report by Stelzner et al. (11) demonstrated evidence of increased pulmonary transvascular protein and water escape within 24–48 h of exposing rats to hypobaric and normobaric hypoxia.

Cellular responses to hypoxia include a redirection of the biosynthetic apparatus with suppression of certain proteins and induction of others (1–8, 54). In our studies, alterations in protein expression appeared to underlie the effects of hypoxia on two endothelial cell surface coagulant properties, suppression of thrombomodulin and induction of a new procoagulant activity not observed in normoxic cultures. Decreased expression of thrombomodulin during hypoxia was a striking finding, since levels were only  $\approx 20\%$  of that observed in controls. Based on the considerable decrease in levels of thrombomodulin mRNA in hypoxic cultures, it seems likely that lower rates of thrombomodulin synthesis contribute to its reduction

under these conditions. Other mechanisms, however, such as shedding of cell surface thrombomodulin or increased degradation, could also be involved. In this context, pilot studies did not demonstrate shedding of thrombomodulin antigen from the surface of hypoxic endothelium using antigenic and functional assays (data not shown). Furthermore, addition of chloroquine to cultures, an agent which has been shown to prevent tumor necrosis factor/cachectin-induced endothelial degradation of thrombomodulin antigen (32), had no effect on hypoxia-induced attenuation of thrombomodulin.

In addition to reduced thrombomodulin, hypoxia induced synthesis and expression of a membrane-associated activator of Factor X. Although the identity of this Factor X activator is unclear, it does not appear to be part of the intrinsic or extrinsic pathways, and most closely resembles the tumor procoagulant (60, 61). The latter enzyme has been purified and shown to be a membrane-associated cysteine proteinase identified in certain malignant tissues. The hypoxic Factor X activator is inhibited by mercury chloride, but not PMSF, which is similar to the tumor procoagulant. Furthermore, both the hypoxic Factor X activator (data not shown) and the tumor procoagulant (61) can be eluted from membranes in the presence of veronal buffer (20 mM, pH 7.8). The  $K_m$  of the enzyme expressed by hypoxic endothelium for the substrate is similar to that of the intrinsic Factor X activation complex and close to the plasma concentration of Factor X (41). However, under the conditions examined in the current experiments  $V_{max}$  is lower with the hypoxic Factor X activator than the Factor IXa-VIIIa complex (41). Although this might suggest that hypoxic endothelium would not be an important contributor to Factor X activation *in vivo*, pilot studies have shown that when the pH is lowered, which frequently occurs with hypoxemia owing to decreased blood flow,  $V_{max}$  increases and  $K_m$  decreases (data not shown). In addition, local hypoxemia and stasis in venous thrombotic disease could potentially favor accumulation of Factor Xa formed by the hypoxic endothelial Factor X activator. Thus, alterations in the endothelial milieu may modulate the effectiveness of the Factor X activator. Consistent with the results obtained *in vitro*, hypoxic native endothelium in aortic segments expressed a Factor X activator and demonstrated decreased thrombomodulin activity.

Although the *in vivo* physiologic relevance of these findings in cell culture has yet to be examined, they may provide a basis for understanding the initiation of vascular pathology under hypoxic conditions, as manifested by increased permeability, as well as a prothrombotic tendency. An association between vessel wall hypoxemia and thrombosis has long been suspected: Sevitt (16) indicated 20 yr ago that thrombi formed in venous valve pockets, an area subject to stasis and hypoxemia (17). Moreover, the observation that the clot was attached to the vessel surface of the valve cusps raised speculation that hypoxia-induced alterations in endothelial cell function could play an important role in the pathogenesis of thrombosis (18). Furthermore, by providing insights into pathways of hypoxic vascular injury, *in vitro* endothelial studies should facilitate the identification and evaluation of protective agents which may ultimately prove useful *in vivo*.

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