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Effect of Hyperoxia on the Cytoarchitecture of Cultured Endothelial Cells

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When confluent pulmonary artery endothelial cells in culture were exposed to hyperoxia (95% O_2 and 5% CO_2), they became enlarged and mean corpuscular volume increased 30–35%. Rhodamine-phalloidin staining of actin filaments demonstrated that hyperoxia was associated with a progressive alteration in the actin distribution. Three days after oxygen exposure, the number and thickness of cytoplasmic stress fibers were increased, while the peripheral bands were disrupted or absent. Sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed that the amount of filamentous actin was increased in oxygen-exposed cells, while the total actin content remained unchanged, suggesting that oxygen exposure shifted the equilibrium from G actin to F actin. (Am J Pathol 1988, 132:59–72)

OXYGEN THERAPY IS an important modality in the treatment of patients suffering from severe hypoxemia. Because of its direct toxic effect on a number of cellular processes, however, it carries with it the risk of exacerbating the underlying lung injury, and may be a contributing factor in death from respiratory failure.[1,2] Ultrastructural studies performed in animals exposed to hyperoxia indicate that the pulmonary capillary endothelium is a major site of oxygen toxicity.[3–5]

Cultured endothelial cells have been used as model systems to study the effect of hyperoxia on the endothelium. A number of events follow the exposure of endothelial cells to high partial pressures of oxygen, including cellular enlargement,[6] enhanced susceptibility to neutrophil oxidant damage,[7] increased neutrophil adherence to endothelial cells, and inhibition of endothelial cell growth in subconfluent cultures.[8] We have demonstrated that exposure of endothelial cell monolayers to hyperoxia for 72 hours results in a significant increase in permeability to ^{125}I albumin.[9] This finding correlates well with the observation that animals exposed to elevated oxygen tensions for 3 days develop endothelial cell swelling, coinciding with interstitial edema, as one of the earliest morphologic indicators of injury.[10]

There is considerable interest in identifying those cellular elements responsible for maintaining endothelial integrity in the face of perturbations that may lead to its destruction. A number of investigators have suggested that endothelial cytoskeletal elements may play such a role, because they participate in maintaining cell shape,[11,12] in the adherence of cells to subcellular matrix[13,14] and in the formation of junctional complexes.[15]

Studies performed in this laboratory on the effects of hyperoxia on albumin permeability of endothelial monolayers have demonstrated marked changes in actin and vimentin filament distribution with oxidant exposure.[9] Other investigators working with endothelium,[16] epithelium,[17] and P388D cells[18] have also noted cytoskeletal alterations in response to oxidant injury.

We have subsequently examined the temporal relationship between oxygen exposure and initiation of size changes as well as cytoskeletal filament distribution and content. Hyperoxia is associated with a time-

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dependent enlargement of endothelial cells. In addition, two distinct patterns of actin filament rearrangements occur with time: an increase in cytoplasmic stress fibers and disruption of peripheral bands.

Materials and Methods

Culture of Endothelial Cells

The established bovine pulmonary artery endothelial (BPAE) cell line (ATCC, CCL-209) was obtained at the 16th passage and used at passages 18 through 24 as described previously.[19] Early passage calf pulmonary artery endothelial (CPAE) cells, 3 through 10 passages, were the gift of Dr. Peter Del Velcchio of the Albany Medical College, and were used for comparative studies. These cells were regarded as endothelial cells based on their characteristic cobblestone morphology and positive staining for factor VIII antigen.[20]

Endothelial cells (BPAE) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY) supplemented with 20% fetal calf serum (FCS) (Hyclone, Logan, UT) and gentamycin 50 μg/ml. (Schering Corp. Kenilworth, NJ) in Corning 75 cm^2 tissue culture flasks (Corning Glass Works, Corning, NY). CPAE cells were cultured in the same media containing 10% serum. For determinations of cell size profiles, 2×10^5 cells/well were seeded into Nunc 24 well plates (A/S Nunc Intermed, Denmark). Cells for cytoskeletal preparations, phase microscopic and immunofluorescence examination were cultured in 35-mm dishes at initial seeding densities of 7×10^5/well. All cultures were incubated at 37 C in a 5% CO_2 incubator for 3–4 days (until confluent) before exposure to oxygen.

Experimental Design for Exposure of Endothelial Monolayer to Oxygen

For each experiment, normoxic and hyperoxic groups were seeded on the same day using cells from the same passage and origin, and allowed to reach confluence. In preparation for oxygen exposure, media was then changed to fresh DMEM supplemented with either 1% FCS (BPAE and CPAE), 10% (CPAE), or 20% (BPAE). These cells were maintained in the 5% CO_2 incubator and 1 group was designated as normoxic controls. Those cells that were to be exposed to 3 days of hyperoxia were immediately placed in an oxygen chamber. Cells to be exposed to 2 days of hyperoxia were placed in oxygen the next day, and 1 day samples were placed in oxygen on the following day. All groups were analyzed on the same day. Thus all cells were exposed to identical growing conditions, the only variable being the time spent in a hyperoxic environment.

For oxygen exposure, endothelial monolayers were placed in an oxygen chamber (Bellco Glass, Inc., Vineland, NJ), flushed for at least 15 minutes with a mixture of 95% O_2 and 5% CO_2, and incubated in a warm room at 37 C. The oxygen chamber was regassed daily or when additional samples were added. Culure medium pO_2 levels were at least 650 torr in hyperoxia-exposed and about 140 torr in control (normoxia-exposed) cultures as measured using a blood gas analyzer (ABL_3 Acid Base Radiometer, Copenhagen, Denmark). The pH of the culture media remained constant (7.35–7.45). In some experiments cells were exposed to either 50% or 80% oxygen as described.

Sizing and Counting of Endothelial Cells

Endothelial cells were counted using an electronic particle counter (Coulter Counter ZM, Coulter Electronics LTD, Hialeah, FL) as described previously.[9] For the total number of endothelial cells, the lower limit of the aperture was set at 9.588 μ so that all particles (cells) with diameters larger than 9.588 μ were counted. Preliminary studies revealed that the cell counts as obtained by the Coulter Counter correlated well with those obtained by the direct cell counting using a hemocytometer.[21] For the purpose of sizing, the number of endothelial cells at various size ranges starting from 9.588 μ in diameter with 2 μ increments were counted. The instrument was calibrated for absolute volume using 20.38 μ microspheres purchased from Coulter Electronics. Recovery of cells by this fractional counting was 102.9 ± 3% (N = 69). Size distribution curves were constructed and the mean diameter and mean corpuscular volume were calculated assuming that endothelial cells in suspension were spherical.

Visualization of Actin Filaments

Actin filaments were visualized in formalin fixed, Nonidet p-40 permeabilized cells as described previously.[22]

Cytoskeletal Extraction and Gel Electrophoresis

Triton X-100 resistant fractions of control and oxygen-treated endothelial cells were prepared by extraction of monolayer cultures in TN buffer (140 mM NaCl, 10 mM Tris-HCl, pH 7.6) containing 1% Tri-

ton X-100 for 5 minutes at 4 C.[23,24] After extraction, the Triton-insoluble residue was scraped into PBS and collected by centrifugation at 13,000*g* for 3 minutes. For determination of total actin and vimentin, monolayers were scraped into Hanks' Balanced Salt Solution (HBSS), collected by centrifugation, then solubilized in Owen-Witte-Baltimore (OWB) buffer[25] that contained 150 mM Tris-Cl, pH 7.4, 0.05% sodium dodecyl sulfate (SDS), 1.0% Na deoxycholate, 150 mM NaCl, and 1.0% Triton X-100. The TN/Triton insoluble pellet and the solubilized total cell extract (1 part extract: 3 parts sample buffer) were dissolved by boiling in electrophoresis sample buffer (0.05 M Tris-Cl, pH 6.8, 10% glycerol, 1% SDS, 1% 2-mercaptoethanol). Proteins were separated by electrophoresis on SDS-9% acrylamide slab gels under denaturing conditions,[26] visualized by staining with Coomassie Brilliant Blue R250 dye, and quantitated by densitometry within the linear range of dye binding sensitivity.[27] Low molecular weight proteins of known mass and purified rabbit skeletal muscle F actin and rat hepatoma vimentin served as standards.

Differential Trypsin/Collagenase Sensitivity Assay

For assessment of the relative substratum adherence of control and oxygen-treated cells, the differential sensitivity to trypsin/collagenase was used as discriminating factor. Trypsin solution was prepared as described above, but supplemented with 0.05% of Type IV-S Collagenase (Sigma Co., St. Louis, MO). Monolayers were washed three times in PBS, after which 1 ml aliquots of trypsin/collagenase solution were added, removed at specific intervals for cell counts, and fresh aliquots added. Detachment of cells was monitored using an Olympus IM2 inverted phase microscope to ensure completeness of cell removal. Data were expressed as the percent of the total population released as a function of time of trypsin/collagenase exposure.

Statistical Analysis

Statistical significance was calculated by the Student's *t*-test.[28]

Results

Effect of Hyperoxia on the Size of Endothelial Cells

As shown in Figure 1, exposure of confluent monolayers of bovine pulmonary artery endothelial cells to oxygen was accompanied by a marked and time-dependent focal swelling of endothelial cells. Oxygen treatment for 24 hours (Figure 1B) did not alter the characteristic cobblestone appearance of the endothelium. However, a number of enlarged cells were evident at 48 hours (Figure 1C) and this feature was more pronounced in 72-hour oxygen-treated cultures (Figure 1D). The presence of enlarged cells conferred an irregular appearance to the endothelial monolayer. Despite the development of cell enlargement, the monolayer appeared to be intact with no obvious gaps seen between cells even after 72 hours of oxygen exposure.

To evaluate this hyperoxia-induced size change quantitatively, monolayers were disrupted by trypsinization and the size distribution of cells determined using an electronic particle counter (Figure 2). Most endothelial cells (more than 80%) were in the size ranges from 13–18 μ in diameter. One day after oxygen exposure, there was no change in the distribution of the cells (Figure 2A). However, after 2 days, the size distribution curve shifted to the right, eg, the number of small cells ($\leq$17 μ in diameter) decreased, while the number of large cells (>17 μ in diameter) increased (Figure 2B). A more pronounced shift was observed after 3 days of exposure. To determine whether cell enlargement also occurred in early passage cells, similar experiments were performed by exposing CPAE to 95% O_2 for 3 days. Table 1 compares sizing data obtained with BPAE and CPAE. Although normoxic control baseline values were smaller for CPAE than for BPAE, oxygen exposure led to a similar magnitude of cellular enlargement, with increases in mean corpuscular volume of 30–35%.

It was of interest to determine whether the size changes seen with 95% O_2 occurred abruptly or could be discerned at lower concentrations of oxygen. This was evaluated by exposing CPAE monolayers either to normoxia or 50, 80, or 95% O_2 for 3 days. For these experiments, cells were maintained in the same concentration of FCS (10%) used for their growth. Table 2 shows that there was no detectable change in cell size at 50%, and although an increase in diameter and volume were apparent at 80%, these changes were not statistically significant. In contrast, there was a clear increase in these parameters at 95% O_2. In addition, comparison of sizing data from 95% oxygen-exposed CPAE in Table 1 (cells exposed to oxygen while in 1% FCS) with that in Table 2 (cells maintained in 10% FCS), demonstrated that cell enlargement was not dependent on the concentration of serum in the media.

Hyperoxia and Actin Filament Distribution

Because of the hyperoxia-associated changes in cell size and monolayer conformation as well as the in-

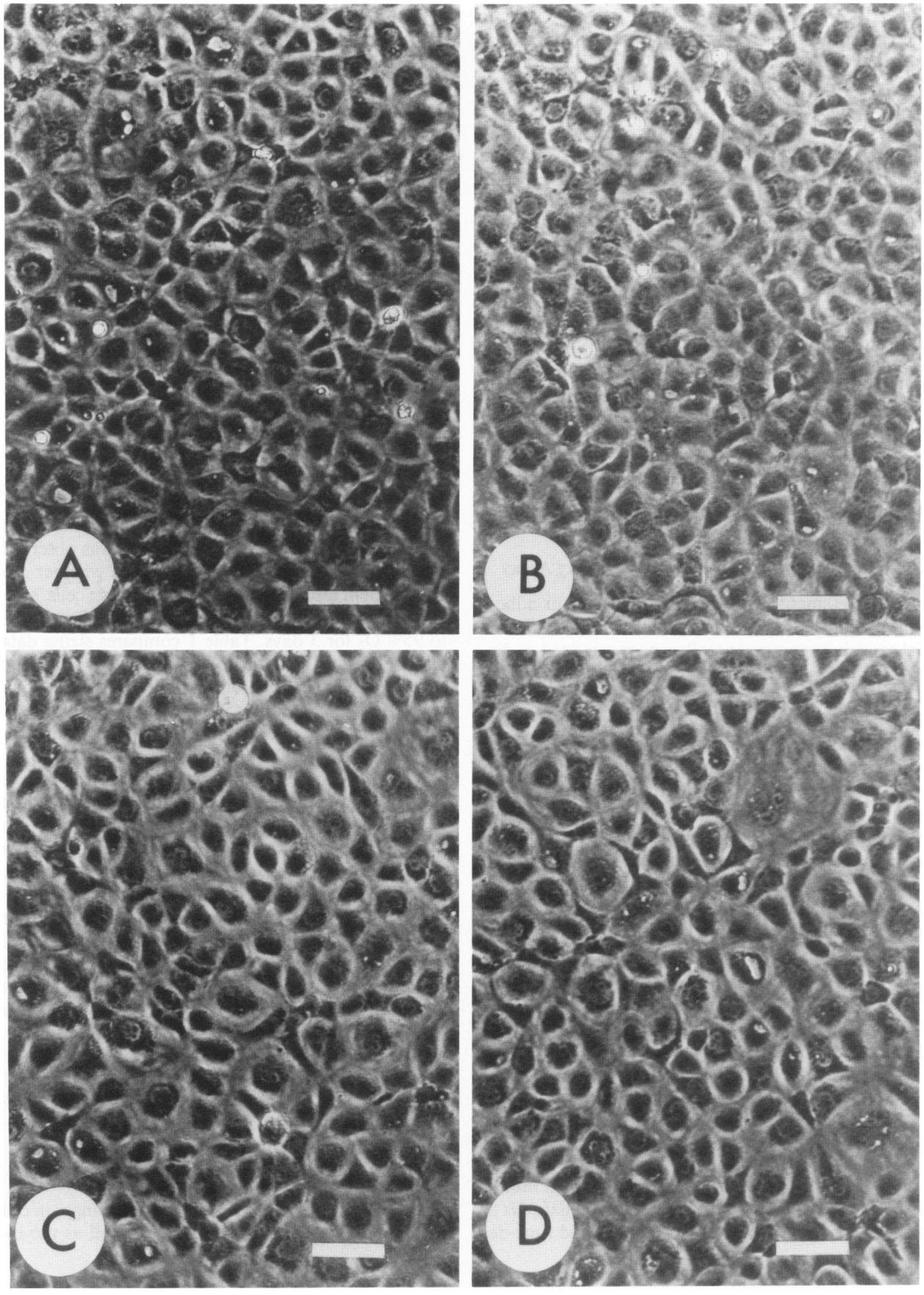
A
B
C
D

Figure 1—Effect of hyperoxia on endothelial cells. Endothelial cells (BPAE) were cultured in DMEM supplemented with 20% FCS for 3 days until confluent. They were then exposed to normoxia or hyperoxia (95% O_2) in DMEM plus 1% FCS for 3 days. The cells were visualized directly by phase microscopy. Normoxia (**A**) Hyperoxia, 1 (**B**) 2 (**C**) or 3 (**D**) days. Bar, 100 μ.

creased albumin permeability described previously,[9] it was important to determine whether these changes in cellular morphology and function were associated with specific cytoarchitectural changes. The distribution of actin filaments in cells exposed to oxygen is shown in Figure 3. Control cells at confluence typically contained very fine anastomosing complexes of microfilaments, with the major structure being a dense peripheral band clearly defining the margins of cells. Few transcytoplasmic stress fibers were visible (Figure 3A). With increasing time in oxygen, BPAE accumulated more transcytoplasmic cables (Figures 3B, C and D). At 3 days, there was an increase in both the number and thickness of stress fibers while the peripheral bands of most cells were disrupted or completely absent (Figure 3D). Although occasional small gaps were seen between adjacent cells in the monolayer after 72 hours of oxygen exposure, this was not a consistent feature and may have represented fixation and staining artifacts. This correlated with the intact monolayer appearance seen by phase microscopy. Changes in monolayer integrity, such as those that result in increased permeability to albumin,[9] may involve more subtle changes (perhaps at the level of cell junctions) than were discernable with phase microscopy. CPAE exposed to 3 days of hyperoxia exhibited identical structural rearrangements (Figure 4).

It was important to determine if enlarged cells exhibited unique actin filament distribution characteristics that might predispose them to act as focal areas for disruption of the monolayer. These features were studied in monolayers exposed to hyperoxia for 3 days, because focal abnormalities are seen more frequently at this time. Enlarged cells exhibited essentially 3 patterns of actin stress fiber distribution (Figure 5): 1) parallel arrays of transcytoplasmic filaments (Figure 5A); 2) stress fibers, particularly in very large cells, which appeared to be fewer and thinner then those of most smaller cells (Figure 5B); and 3) Extensively bundled discontinuous patches of actin stress fibers (Figures 5C and D). These patterns were, for the most part, not unique to the enlarged cells and similar patterns were seen in smaller cells within the oxygen-exposed monolayer. There did not appear to be any preferential loss of peripheral bands in enlarged cells or in cells adjacent to enlarged cells. Rather, the thinning or discontinuity within the cortical actin filament structure seemed to be common to most cells within the monolayer at 3 days. Identical results were obtained when cells were cultured in 20% FCS/

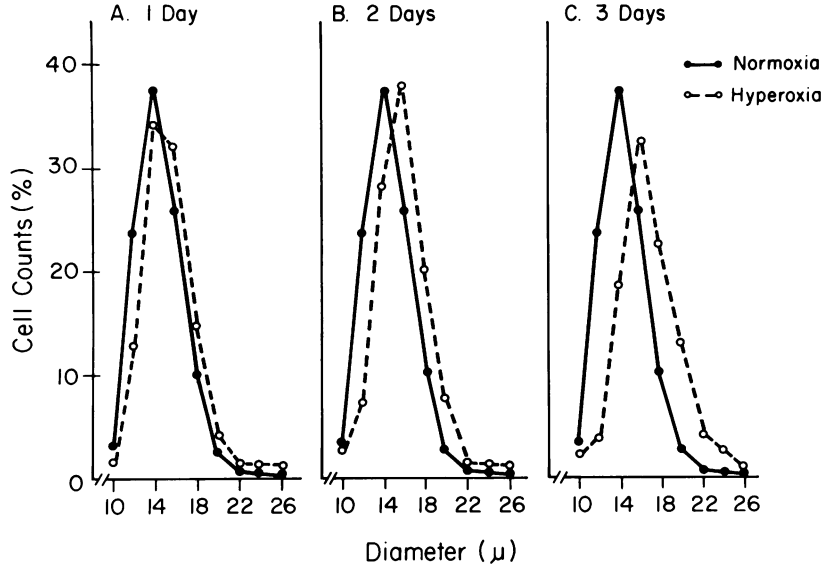

Figure 2—Effect of hyperoxia on the size distribution of endothelial cells. Endothelial monolayers (BPAE) were cultured and exposed to normoxia or hyperoxia (95% O_2) as described in Figure 1 for 1–3 days. Endothelial cells were then removed by trypsinization and sized using an electronic particle counter (Coulter Counter ZM). Results are from a representative experiment.

Table 1—Effect of Hyperoxia on the Size of Endothelial Cells

	BPAE	CPAE
Mean diameter (μ)		
Normoxia	15.43 ± 0.22	13.88 ± 0.15
Hyperoxia	17.09 ± 0.30*	15.16 ± 0.12*
Mean corpuscular volume (fl)		
Normoxia	1,927 ± 48	1,400 ± 46
Hyperoxia	2,617 ± 141*	1,824 ± 44*
Percent increase in volume	35.8	30.3
N (number of experiments)	6	4

Endothelial cells were cultured in DMEM supplemented with either 20% FCS (BPAE) or 10% FCS (CPAE) for 3 days until confluent. They were then exposed to normoxia or hyperoxia (95% O_2) in DMEM plus 1% FCS for 3 days. Endothelial cells were trypsinized and their size distribution determined using an electronic particle counter. Mean diameter and mean corpuscular volume were calculated assuming that endothelial cells in suspension were spherical. Results were expressed as mean ± SEM.

* P (*vs.* normoxia) <0.02.

DMEM for oxygen exposure (data not shown), indicating that the observed cytoskeletal alterations were not dependent on serum concentration.

It was of interest to determine whether the marked changes in actin filament distribution occurred to the same extent in cells exposed to lesser concentrations of oxygen. The results of exposure to normoxia or to 50, 80, or 95% O_2 for 3 days are shown in Figure 6. Cells exposed to 50% oxygen, exhibit few, or only subtle changes (Figure 6B) in comparison to controls (Figure 6A). However, cells exposed to 80% (Figure 6C), demonstrated some of the features characteristic of exposures to 95% oxygen (Figure 6D). There was an increase in the number of stress fibers, enlarged cells were evident. While peripheral bands appeared intact in some cells, a number of them demonstrated disruption or loss of this structural feature (Figure 6C).

SDS-PAGE Analysis of Cytoskeletal Proteins

Gel electrophoretic analysis of cytoskeletal protein extracts (Figure 7) was performed to quantify the apparent increased content of actin filaments observed morphologically. Data compiled from 4–6 experiments in Table 3 indicated that exposure to oxygen results in quantitative increases in cellular filamentous actin. Such increases were occasionally apparent as early as 24 hours, however, due to variability within gels at this time period, significance was not achieved until 48 hours. The content of vimentin, the major intermediate filament protein of endothelial cells also exhibited significant increases in hyperoxia-exposed cells.

The above results indicated that hyperoxia was associated with an increase in the amounts of filamentous (detergent resistant) actin and vimentin in endothelial cells. This could be due to an increase in the total actin and vimentin contents. Alternatively, this could be due to an altered distribution of actin and vimentin, while their total cellular contents remained unchanged. To distinguish these possibilities, we quantified the total actin and vimentin contents. As shown in Table 4, hyperoxia had no effect on the total actin or vimentin contents of endothelial cells.

Differential Trypsinization

During the course of our experiments, especially those that required removal of oxygen-treated cells from dishes for analysis, it became apparent that these cells were more adherent than their normoxic counterparts. Furthermore, both morphologic and quantitative data on actin filament distribution had indicated substantial increases in cytoplasmic actin stress fibers. Actin stress fibers have been shown to participate in the formation of focal adherence contacts.[13,14] To quantify these apparent differences in substrate adherence directly, cells from each group were analyzed for differential sensitivity to trypsin/collagenase detachment. Figure 8 shows the results of a representative experiment (total of 4 experiments) performed after 72 hours of oxygen exposure, indicating a clear separation in the kinetics of cell detachments for normoxic and hyperoxic cells. Under normoxic condi-

Table 2—Effect of pO_2 on the Size of Endothelial Cells

	Normoxia	50% O_2	80% O_2	95% O_2
Mean diameter (μ)	13.86 ± 0.08	13.82 ± 0.05	14.15 ± 0.13	15.23 ± 0.27*
MCV† (fl)	1394 ± 24	1382 ± 15	1483 ± 42	1850 ± 99*
Percent change in volume		−0.86	+6.4	+32.7
N	12	6	6	12

Endothelial cells (CPAE) were cultured in DMEM supplemented with 10% FCS for 3 days until confluent. They were then exposed to normoxia or hyperoxia (either 50, 80, or 95% O_2) in DMEM plus 10% FCS for 3 days. Cells were trypsinized and their size distribution determined using an electronic particle counter. Results were expressed as mean ± SEM.

* P (*vs.* normoxia) <0.01.

† MCV, mean corpuscular volume.

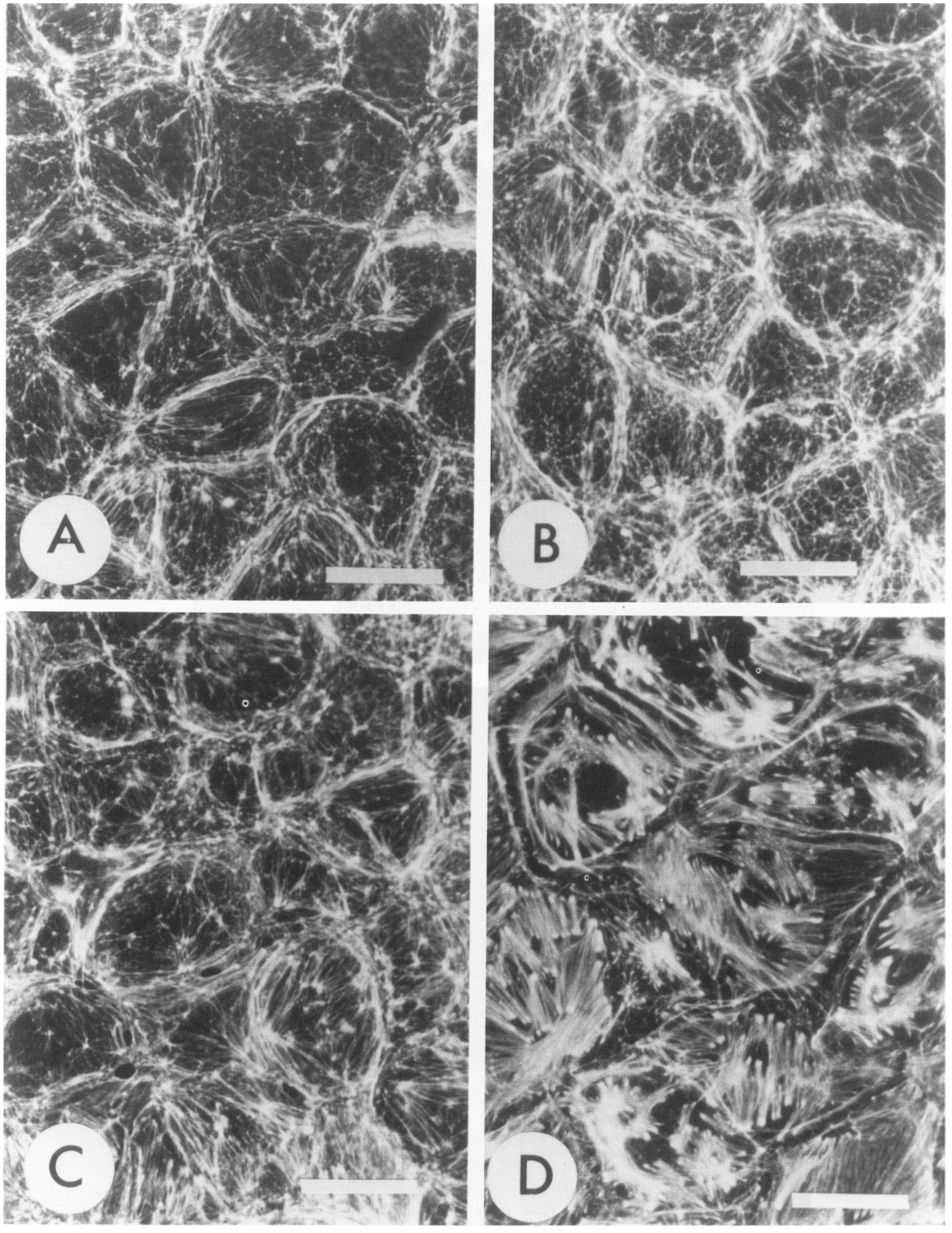

Figure 3—Effect of hyperoxia on actin filament distribution in endothelial cells. Confluent monolayers (BPAE) were exposed to normoxia (**A**) or hyperoxia (95% O_2) for 1 (**B**), 2 (**C**) or 3 (**D**) days in 10% FCS/DMEM. Cells were stained with rhodamine phalloidin and examined microscopically. Bar, 50 μ.

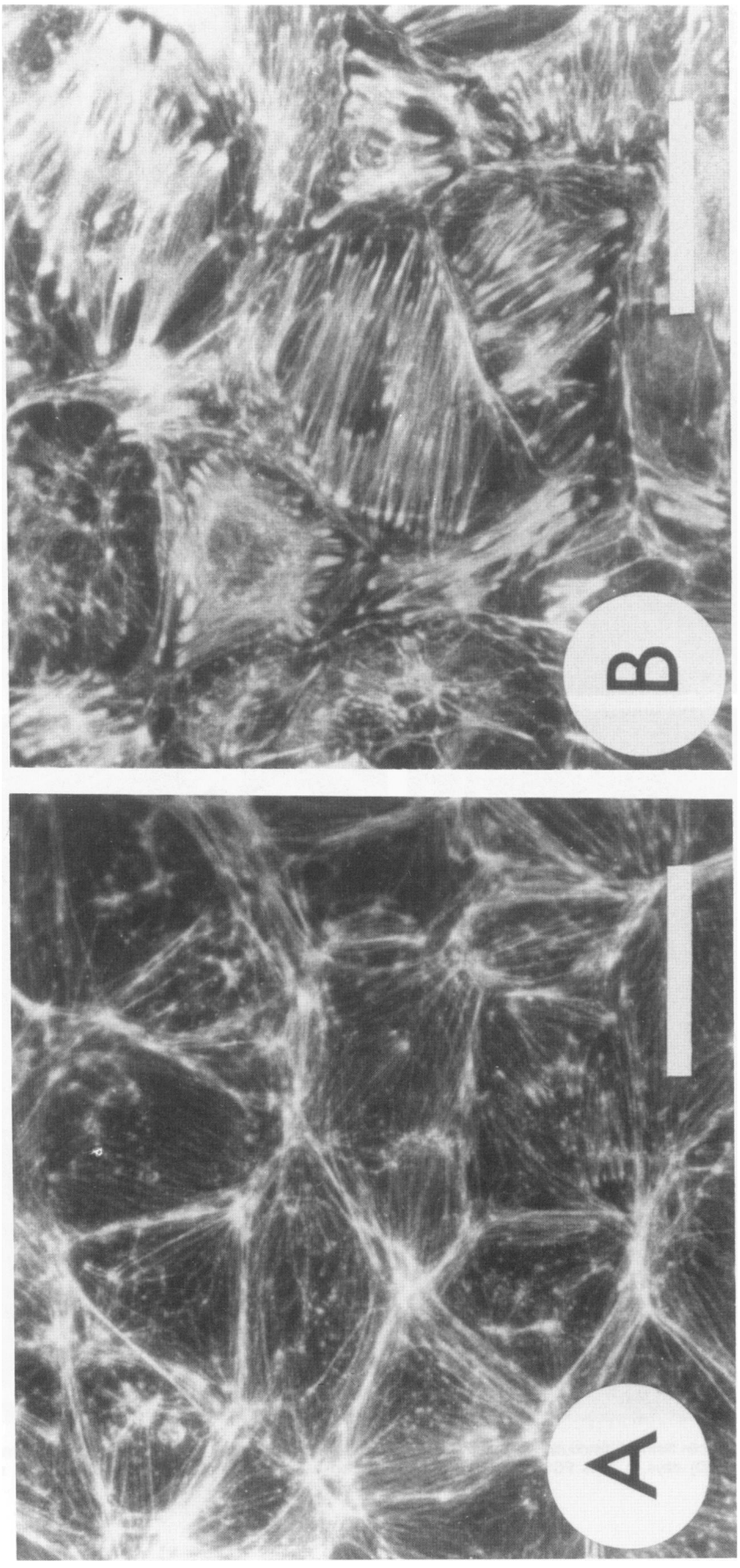

Figure 4—Effect of hyperoxia on actin filament distribution in endothelial cells. Confluent CPAE monolayers cultured in 10% FCS/DMEM were exposed to normoxia (**A**) or hyperoxia (**B**) for 3 days. Cells were stained with rhodamine phalloidin and examined microscopically. Bar, 50 μ.

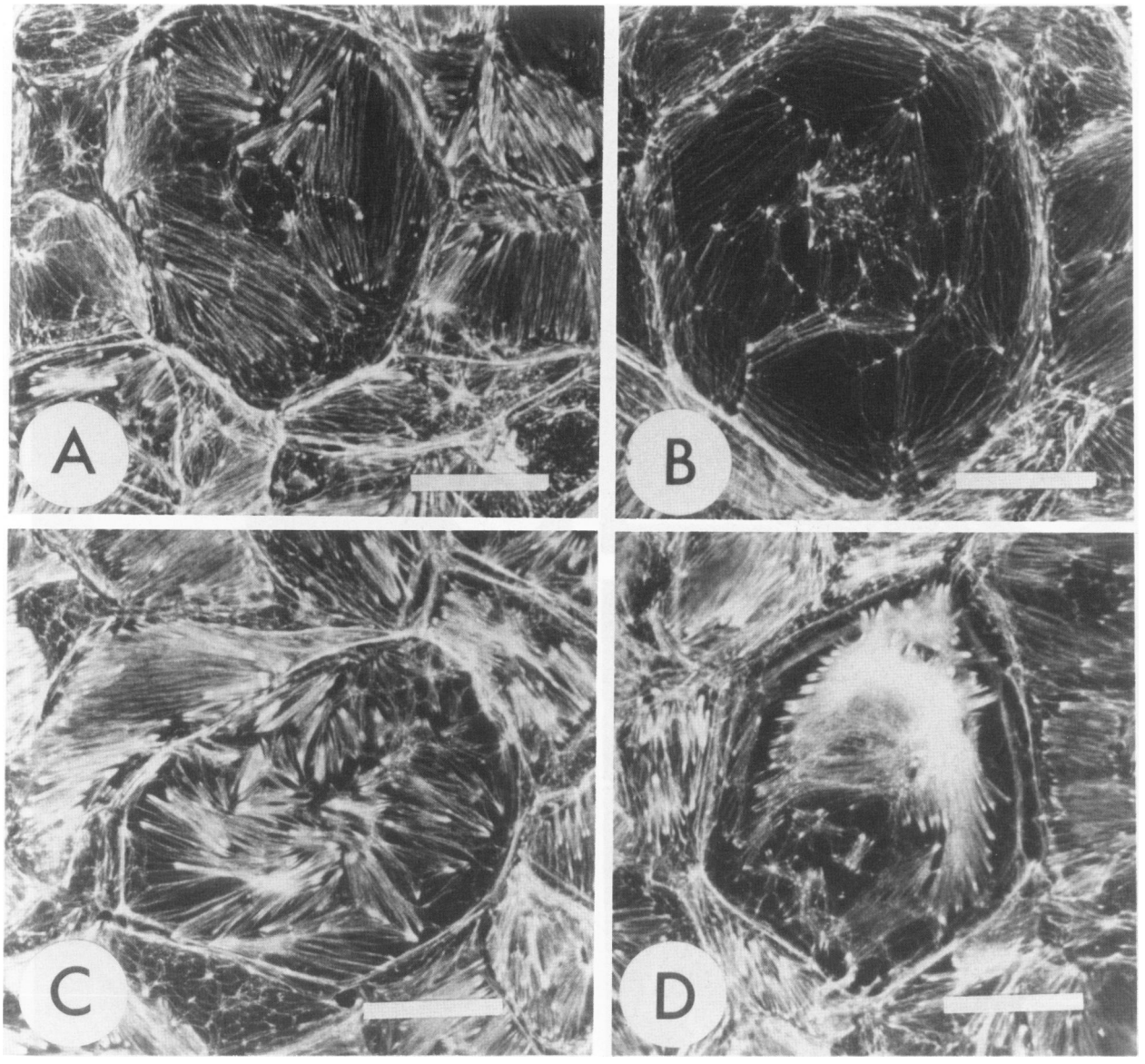

Figure 5—Effect of hyperoxia on actin filament distribution in enlarged endothelial cells. Confluent (BPAE) monolayers were exposed to hyperoxia (95% O_2) for 3 days in 1% FCS/DMEM then stained with rhodamine phalloidin and examined microscopically. **A**—Enlarged cell shows parallel array of filaments similar to smaller cells surrounding it. **B**—Cell has stress fibers which appear to be thinner than those of other cells. **C and D**—Cells exhibiting extensively bundled, discontinuous patches of stress fibers. Bar, 50 μ.

tions, a cumulative total of 80% of the cells had detached by 12 minutes, in comparison to 42% of hyperoxia-exposed cells. A shift in the curve to the right for hyperoxic cells indicated the presence of a trypsin/collagenase resistant population that required 18 minutes for complete removal.

Discussion

Enlargement of endothelial cells in response to hyperoxia has been demonstrated previously in animals exposed to elevated partial pressures of oxygen,[4,10] as well as *in vitro*.[6] We have used an electronic particle counter, as described by others[29] to size endothelial cells. This permitted the determination of cell sizes within the entire cell population. Although direct size measurements of attached cells were not performed, matched control and hyperoxic samples were exposed to the same trypsinization conditions in order to determine relative size distributions. Clear differences, reflecting the morphologic observations, were seen at 48 and 72 hours at 95% oxygen. These hyperoxia-as-

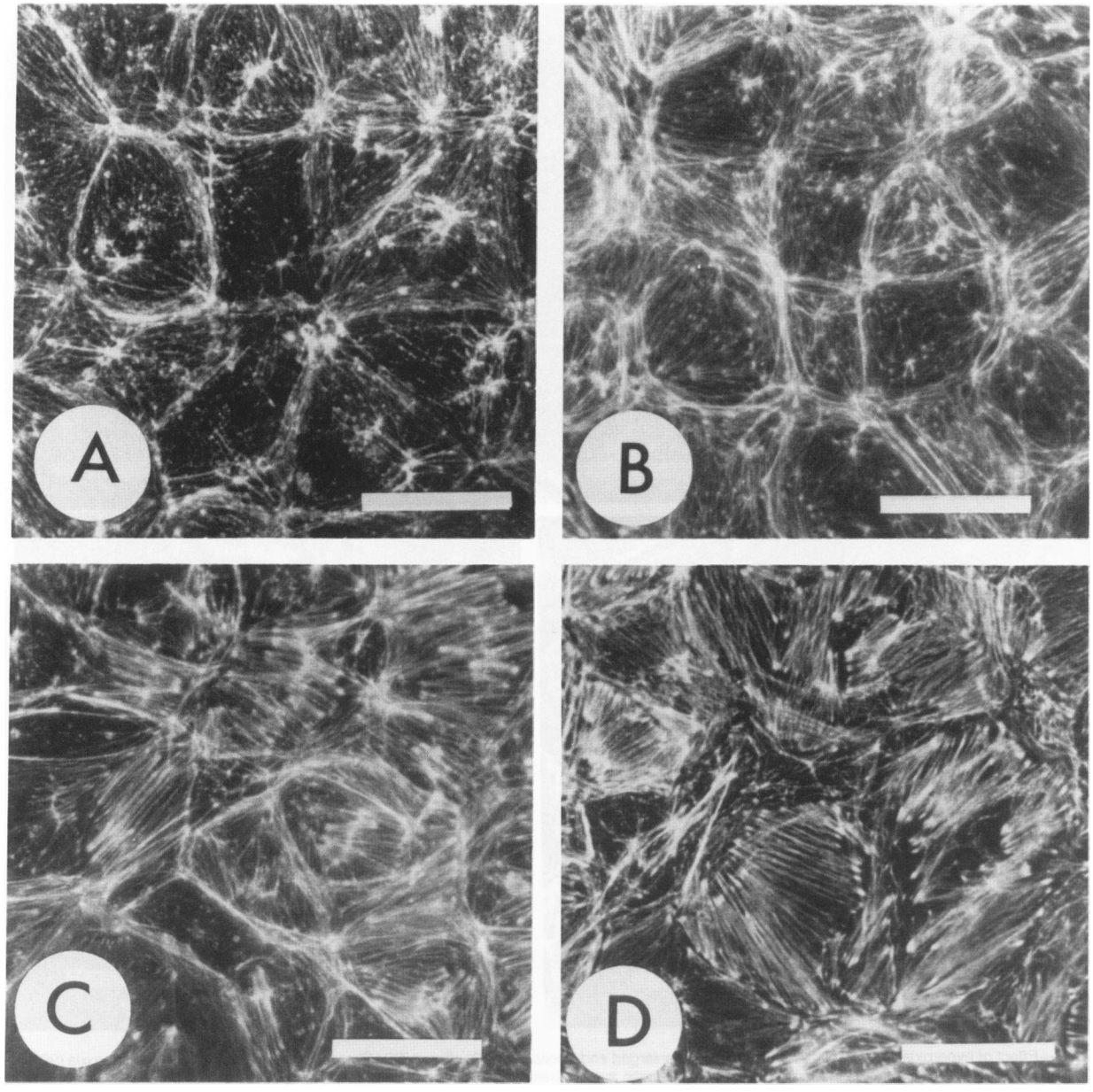

Figure 6—Effect of oxygen concentrations on actin filament distribution in endothelial cells. Confluent BPAE monolayers cultured in 20% FCS/DMEM were exposed to normoxia (**A**) or hyperoxia at 50% (**B**), 80% (**C**), or 95% (**D**) for 3 days. Cells were stained with rhodamine phalloidin and examined microscopically. Bar, 50 μ.

sociated size changes are independent of FCS concentrations in the culture media or passages of endothelial cells.

Specific cytoskeletal alterations also occur when endothelial cells are exposed to hyperoxia. Oxygen exposure resulted in two distinct cellular response patterns involving actin filament distribution. Transcytoplasmic cables increased both in number and in thickness with increasing oxygen exposure. These apparent increases in actin microfilaments were confirmed by polyacrylamide gel electrophoresis of detergent-insoluble, cytoskeleton-enriched fractions. It is believed that, for cultured cells[13,14] and in some instances *in vivo*,[30] these stress fibers serve an anchoring function, maintaining the monolayer firmly attached to the substratum. The increase in the time of trypsin/collagenase exposure required to detach oxygen-treated cells that had developed an extensive transcytoplasmic cable network supports this hypothesis. Although it is not possible at this time to rule out oxidative

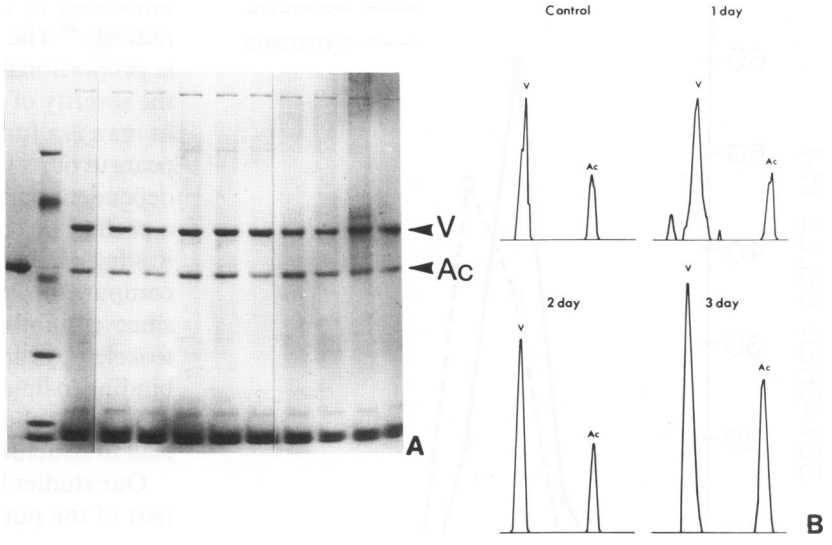

Figure 7—Increase in cytoskeletal-associated actin (Ac) and vimentin (V) content in BPAE cells as a consequence of time of oxygen (95%) exposure. Within each experimental group (1–3; panel A), the detergent-resistant cytoskeletal residue from equivalent number of control or oxygen-treated cells were solubilized by boiling in aliquots of Laemmli sample buffer, equivalent volumes loaded per lane, and the constituent proteins separated on SDS-9% acrylamide slab gels; individual peptides were visualized by staining with Coomassie Blue R250. Lane designations (in A) are as follows: (a) actin standard; (b) molecular weight protein standards – phosphorylase B = 93 kd, bovine serum albumin = 68 kd, ovalbumin = 43 kd, carbonic anhydrase = 31 kd, soybean trypsin inhibitor = 22 kd; (c, d, e) experiment 1, 72 hour oxygen, 24 hour oxygen, normoxia control, respectively; (f, g, h) experiment 2, 72 hour oxygen, 48 hour oxygen, 24 hour oxygen, respectively; (i, j, k, l) experiment 3, 72 hour oxygen, 48 hour oxygen, 24 hour oxygen, normoxia control, respectively. Quantitative scanning densitometric analyses of electrophoretic separations (panel B; data from experiment 3 is illustrated as one example) revealed significant increases in the vimentin and actin contribution to the cytoskeletal-associated protein fraction of BPAE cells as a function of oxygen exposure.

changes in other components of the cell-to-substrate junctional complex which might influence adherence, these data suggest that one aspect of the cells' adaptive machinery may include alterations in the actin cytoskeleton which favor substrate anchorage.

While stress fibers are commonly seen in some types of cells in culture, they are observed *in vivo* in only a few settings. They occur in arterial endothelial cells in experimentally induced[31] or spontaneous hypertension,[32] in myofibroblasts involved in wound healing,[33] in regenerating liver, and in several human carcinomas,[34] and in cells exposed to high shear stress such as in the aortic arch or in heart valves.[32,35] Increases in stress fibers in cultured cells can be elicited by exposing the cells to increasing shear force simulating the *in vivo* condition.[36,37] These perturbations result in extensive reorganization of both F actin filaments and in the cellular fibronectin network beneath the cell monolayer.[37] From the results of our analysis of total actin in cells exposed to hyperoxia, it seems likely that since there does not appear to be more total actin, the increase in the number and thickness of filaments may result from a shift in the equilibrium from G actin to F actin in these cells. Similar shifts in equilibrium favoring F actin are also observed in tumor cells and regenerating liver,[34] and in migrating cells.[38,39]

We have consistently observed that 3-day oxygen exposure leads to disruption of the peripheral band of actin filaments.[9] Although both cytoplasmic and cortical actin filaments are specifically stained with phalloidin, they exhibit different sensitivities to disruptive agents such as cytochalasin[40] and ethchlorvynol,[41] and appear to serve different functions within the cell. The dense peripheral bands in endothelial cells share many similarities with the circumferential band of mi-

Table 3—Effect of Hyperoxia on the Cytoskeletal Proteins of Cultured Endothelial Cells

Duration of exposure	Filamentous actin (% control)	Filamentous vimentin (% control)
1 day (N = 4)	136 ± 41	149 ± 23
2 days (N = 6)	149 ± 16*	129 ± 17
3 days (N = 6)	212 ± 39*	197 ± 34*

Confluent endothelial cell monolayers were extracted with TN/Triton buffer at 4°C. The detergent insoluble pellets were solubilized by boiling in electrophoresis sample buffer and run on 9% SDS-PAGE. Coomassie blue stained bands were quantitated by scanning densitometry. Results are expressed as mean ± SEM.

* P (*vs.* Control) <0.05.

Table 4—Effect of Hyperoxia on the Total Contents of Actin and Vimentin in Cultured Endothelial Cells

Duration of exposure	Actin (% control)	Vimentin (% control)
1 day	127	114
2 days	103	95
3 days	100	102

Confluent endothelial cell monolayers were scraped into HBSS, collected by centrifugation and the pellet solubilized with OWB buffer. An aliquot was then boiled (1:3) with electrophoresis sample buffer and run on 9% SDS-PAGE. Coomassie blue stained bands were quantitated by scanning densitometry.

The results were the mean of 2 independent experiments.

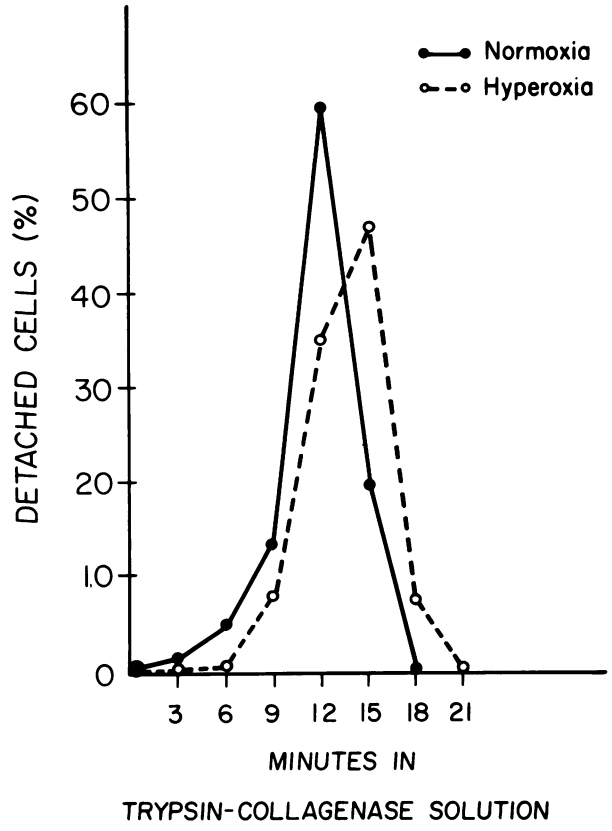

Figure 8—Effect of hyperoxia on trypsin/collagenase sensitivity of endothelial monolayers. Cells were exposed either to normoxia or hyperoxia (95% O_2) for 3 days. Monolayers were treated with trypsin/collagenase solution and the number of cells which detached at specific intervals was determined. Results are from a representative experiment.

crofilaments seen in the apical portion of epithelial cells.[42-44] These cells form tight monolayers where the apical actin bundles are associated with the vinculin adhaerens junction present at the interface between adjacent cells in the monolayer.[42,43] It has been proposed that the vinculin plaques observed in endothelial cells may be part of a junction similar to the adhaerens junction.[15] It is interesting that the microfilament-adhaerens junction complex has been demonstrated to regulate permeability across epithelial monolayers.[45,46] Thus loss or disruption of this crucial structural element may compromise the integrity of the endothelial monolayer as manifested by increased permeability. Two agents that preferentially target actin peripheral bands in endothelial cells, cytochalasin and ethchlorvynol, have been shown to lead to increased lung wet weight[40] and reversible pulmonary edema,[47] respectively.

It is interesting that alterations in actin filament distribution appear to be dependent upon the partial pressure of oxygen. Exposure to 50% oxygen elicited only subtle changes, while progressive disruption was observed at 80% and 95%. Oxygen toxicity is generally attributed to the intracellular generation of oxygen radicals.[48] The concentration of these oxygen radicals is proportional to the pO_2 of the media.[49] Likewise, the severity of oxidant damage in animal models and in man is a function of the oxygen level used.[50] It appears in our studies that cytoarchitectural changes are dependent on this factor as well. Whether this is a direct effect on actin filaments or whether they are manifesting changes occurring in some other structural component remains to be determined. For example, since a number of calcium-dependent cortical cytoskeletal proteins are linked to the membrane by binding to lipid molecules,[51,52] oxidation of lipid molecules may alter the character of the bond directly and lead to structural disruptions.

Our studies have addressed only actin filaments as part of the putative adhaerens complex. It is reasonable to assume that other actin binding proteins such as vinculin, α-actinin, and myosin may also be altered in the course of oxygen exposure. It seems unlikely from our observations of actin filaments in endothelial cells with progressive O_2 exposure that enlarged cells act as foci for weakening of cell–cell contacts, a feature that may be necessary for increased permeability to macromolecules. Rather, this disruption of cortical actin filaments appears to be a common feature seen in most cells after 72 hours. Investigations of other proteins that stabilize the membrane skeleton and may participate in maintaining endothelial barrier function are currently under way in this laboratory.

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