Effect of hypercarbia on surface proteins of cultured bovine endothelial cells

SHARON ROUNDS,1 DAMANI PIGGOTT,1 DOLORETTA D. DAWICKI,1 AND HARRISON W. FARBER2

1Pulmonary and Critical Care Section, Providence Veterans Affairs Medical Center, Brown University School of Medicine, Providence, Rhode Island 02908; and 2The Pulmonary Center, Boston University School of Medicine, Boston, Massachusetts 02118

Rounds, Sharon, Damani Piggott, Doloretta D. Dawicki, and Harrison W. Farber. Effect of hypercarbia on surface proteins of cultured bovine endothelial cells. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L1141–L1146, 1997.—Hypercarbia is a common complication of respiratory failure, and the technique of “permissive hypercapnia” is used to ventilate individuals with increased peak airway pressures on mechanical ventilators, resulting in elevated arterial PCO2. We studied the effects of hypercarbia on cultured bovine aortic and main pulmonary artery endothelial cell surface proteins, assessing cell surface iodination using lactoperoxidase bound to latex microspheres. We found that 4 h of exposure to 10% CO2 increased the display of substances of apparent molecular masses of 27, 47, and 52 kDa. This effect was not mimicked by acidotic media. Western blots of detergent extracts of main pulmonary artery endothelial cell monolayers did not show increased expression of carbonic anhydrase IV (molecular mass = 52 kDa) after incubation under hypercarbic conditions. Hypercarbia did not change the pattern of [35S]methionine incorporation into endothelial cell proteins. We conclude that hypercarbia of 4-h duration changes iodinated endothelial cell surface proteins. We speculate that this effect may be related to changes in secretion or display of apical cell membrane-associated proteins.

HYPERCARBIA AND HYPOXIA are common complications of respiratory failure. In addition, the technique of “permissive hypercapnia” is used to ventilate individuals with increased airway pressures on mechanical ventilators, resulting in elevated arterial PCO2. This strategy for mechanical ventilation has been reported to be useful in reducing peak airway pressures in patients with bronchospasm and in reducing plateau pressures in patients with acute respiratory distress syndrome, thereby decreasing the incidence of barotrauma and volutrauma lung injury (24). Acute and/or chronic hypoxia can change endothelial cell protein synthesis with production of stress-related proteins (7, 30), tropomyosin (18), endothelin (12), and platelet-derived growth factor B (11). Although the effects of acute (within minutes) elevation of CO2 on endothelial cell intracellular pH and Ca2+ influx (29), nitric oxide release (8), and prostaglandin production (9) have been reported, little is known regarding the effects of hypercarbia on endothelial cell protein expression.

Because the luminal surface of endothelial cells is exposed to hypoxic and hypercarbic blood in patients with respiratory failure and because the endothelial cell surface influences blood coagulation (17) and leukocyte adhesion (6), we studied the effects of short-term hypoxia and hypercarbia on cultured aortic and pulmonary artery endothelial cell surface proteins. We studied cell surface iodination using soluble lactoperoxidase and lactoperoxidase bound to latex microspheres. We found that 4 h of exposure to 10% CO2 increased the display of substances of apparent molecular masses of 27, 47, and 52 kDa. Because the molecular mass of bovine lung carbonic anhydrase IV has been reported to be 52 kDa (26), we assessed the effects of hypercarbia on main pulmonary artery endothelial cell carbonic anhydrase IV. Western blots of detergent extracts of main pulmonary artery endothelial cell monolayers did not show increased expression of carbonic anhydrase IV after incubation under hypercarbic conditions. These results suggest that exposure to increased environmental CO2 may alter the display of endothelial cell surface proteins.

MATERIALS AND METHODS

Materials. Enzymobeads were obtained from Bio-Rad (Richmond, CA) and consist of lactoperoxidase and glucose oxidase immobilized on polystyrene latex spheres <0.7 µm in diameter. Lactoperoxidase and glucose oxidase were obtained from Calbiochem (San Diego, CA), and Na125I, [35S]methionine, and Na235CrO4 were obtained from Amersham (Arlington Heights, IL).

Polyclonal rabbit anti-bovine and anti-rat lung carbonic anhydrase IV antisera were kindly donated by Drs. Abdul Waheed and William Sly of Washington University, St. Louis, MO (5, 26).

Endothelial cell cultures. Bovine main pulmonary artery endothelial cells (BPAEC) and bovine aortic endothelial cells (BAEC) were isolated using a standard scraping technique and were cultured in minimal essential medium (MEM) plus 12% fetal calf serum (FCS) as previously described (14, 19). Endothelial cells were identified by a characteristic “cobblestone” appearance by phase microscopy, factor VIII staining, and uptake of acetylated low-density lipoprotein.

CO2 exposures. Confluent cultures of BAEC or BPAEC in 35-mm dishes were incubated for 4 h in air-tight chambers at 37°C in MEM plus 12% FCS in atmospheres with varying gas tension: 0% O2-5% CO2, 0% O2-10% CO2, 21% O2-5% CO2, or 21% O2-10% CO2. The chambers were flushed with the gas mixtures for 5 min and then were sealed. After incubation, monolayers were washed three times with phosphate-buffered saline (PBS) and were prepared for lactoperoxidase iodination, as described in Lactoperoxidase labeling, or were extracted for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and enhanced chemiluminescence (ECL) - Western blotting as described in ECL-Western immunoblots.

In other experiments, adherent cell counts were performed with a hemocytometer after release of cells from the monolayer.
ers with trypsin, and samples of media were obtained for determination of pH and O\textsubscript{2} and CO\textsubscript{2} tensions. In addition, some monolayers were preincubated with Na\textsubscript{251}CrO\textsubscript{4} in MEM overnight before gas exposures. After gas exposures, \textsuperscript{51}Cr release was determined as a measure of cell viability as previously described (14).

To determine the effect of acidosis alone, in some experiments, cultures were incubated for 4 h at 37°C in 95% air-5% CO\textsubscript{2} in MEM plus 12% FCS, the pH of which had been adjusted to 7.10 or 7.30 by the addition of 0.1 N HCl or 0.1 N NaOH. After incubation, the cultures were iodinated using the bead method, and surface proteins were analyzed as described in Lactoperoxidase labeling. Media were collected and immediately analyzed for pH values, which were 7.38 (pH 7.3 culture) and 7.01 (pH 7.1 culture).

Lactoperoxidase labeling. After exposure to CO\textsubscript{2} or acidosis, monolayers were iodinated for 15–30 min at 23°C using 0.25 µCi Na\textsuperscript{125}I and 10 mM glucose in PBS with either 100 µl of Enzymobeads or 100 µl of 7.4 U/ml lactoperoxidase plus 150 µl of 0.2 U/ml glucose oxidase. Iodinated monolayers were washed three times with PBS, then extracted with 30 mM n-octyl glucoside in 10 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8) for 20 min, and centrifuged at 12,000 g for 3 min. The supernatant fraction (cytosol and membranes) was 85 ± 0.5% of total cell protein (n = 4). Aliquots of detergent extract supernatants containing 500,000 counts/min were separated using SDS-PAGE, unreduced, and reduced with 10 mM dithiothreitol.

\textsuperscript{[35S]}methionine labeling. In other experiments confluent monolayers of BAEC were incubated for 4 h in atmospheres of varying gas tension in methionine-free MEM supplemented with 45 µCi \textsuperscript{[35S]}methionine. At the end of the incubation period, media were collected, and monolayers were washed three times with PBS. Protein was precipitated from media with 10% trichloroacetic acid. Cells were extracted with 30 mM n-octyl glucoside in 10 mM Tris·HCl (pH 8) for 20 min and were centrifuged at 12,000 g for 3 min. Aliquots of detergent extract supernatants and solubilized media proteins were separated by SDS-PAGE with loading of equal counts into each lane.

ECL-Western immunoblots. After incubation in varying gas tensions for 4 h, cultures were washed two times in PBS, and cells were lysed with 2% SDS in 62.5 mM Tris and 1 mM EDTA with or without 40 mM dithiothreitol at pH 6.8 for 30 min. Lysates were centrifuged at 12,000 g for 5 min, and supernatants were assayed for protein using the Bio-Rad assay. Samples were subjected to electrophoresis using a 5–15% polyacrylamide gel with equal loading of protein into all lanes. The proteins were electrophoretically transferred to Immobilon (Millipore, Marlborough, MA) and were incubated for 1 h in blocking buffer containing 5% serum albumin. The blot was rinsed three times with rinsing buffer containing 20 mM Tris·HCl, 150 mM NaCl, 0.5% (vol/vol) Tween 20, and 5% bovine serum albumin, incubated at room temperature for 2 h with rabbit anti-bovine or anti-rat polyclonal antibody to carbonic anhydrase IV, and diluted 1:1,000 in rinsing buffer containing 5% nonfat dry milk. At the end of the incubation period, the blot was again rinsed with rinsing buffer and was reincubated for 1 h in a solution containing protein A conjugated to horseradish peroxidase diluted 1:1,000 with rinsing buffer. Protein A detection was done by the ECL method as described by the manufacturer (Amersham).

RESULTS

Figure 1 shows autoradiographs of gels after endothelial cells had been incubated in varying gas tensions for 4 h followed by iodination using immobilized lactoperoxidase-glucose oxidase. Figure 1A shows that inco...
tion of main pulmonary artery endothelial cells under conditions of 10% CO$_2$ caused dramatic increases in labeling of bands of apparent molecular masses of 52 and 27 kDa. Less impressive increases were also noted for a 47-kDa band. The increases in the 47- and 52-kDa bands were observed in gels run under nonreduced conditions but not consistently under reducing conditions. The increases in bands at 52 and 27 kDa were confirmed by densitometry, which demonstrated that the 52-kDa band increased 3.1-fold when exposed to 10% CO$_2$ under hypoxic conditions and increased 11.8-fold when exposed to 10% CO$_2$ under normoxic conditions. The 27-kDa band increased 2.5-fold when exposed to 10% CO$_2$ under hypoxic conditions and 5.8-fold when exposed to 10% CO$_2$ under normoxic conditions. Figure 1B shows similar results for BAEC. These results indicate that exposure to hypercarbia for 4 h changed cell surface proteins in that at least three substances displayed enhanced labeling.

Four hours of exposure to 0% O$_2$-5% CO$_2$, compared with 21% O$_2$-5% CO$_2$, appeared to enhance the display of bands at 27, 47, and 52 kDa (Fig. 1, A and B). Thus the degree of increase in the density of these bands caused by 10% CO$_2$ was less under hypoxic conditions. The effect of hypoxia on the display of the bands at 27, 47, and 52 kDa was much less dramatic than that of hypercarbia. Nevertheless, it is possible that there is some interaction between hypoxia and hypercarbia in endothelial cell surface protein display.

When endothelial cells were iodinated using soluble lactoperoxidase, neither hypoxic nor hypercarbic conditions altered the pattern of surface iodination (Fig. 2). These results indicate that hypercarbia selectively changed the iodination of cell surface proteins that were accessible to latex microspheres, such as apical cell surface proteins.

The effects of varying gas tensions on adherent cell counts, $^{51}$Cr release, and media pH and gas tensions are shown in Table 1. Endothelial cells were not injured by either hypoxia or hypercarbia as assessed by adherent cell counts and $^{51}$Cr release.

Table 1 indicates that hypercarbic cells were acidotic in bicarbonate-based buffer. To determine whether extracellular acidosis was the cause of altered apical surface iodination, we incubated cells in MEM that had been titrated to pH 7.10 or 7.30 before 4 h of incubation in 21% O$_2$-5% CO$_2$. Figure 3 shows that extracellular acidosis per se did not increase surface iodination of BAEC when iodination was assessed using lactoperoxidase conjugated to beads. These results indicate that the effect of CO$_2$ on apical surface iodination was not due to changes in media pH. However, the cells exposed to acid media did have a decreased display of iodinated substances of apparent molecular masses of 46 and 190 kDa.

Because of the possibility that surface iodination changes might be caused by effects of hypercarbia on serum proteins adsorbed to endothelial cells, we assessed the effects of 4 h of incubation in varying gas tensions on iodination of 15% FCS in MEM alone in the absence of endothelial cells. We found no effects of 10% CO$_2$ on iodination of FCS alone (data not shown).

Table 1. Effect of varying gas tensions on media pH and gas tensions, adherent cell counts, and $^{51}$Cr release

<table>
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<tr>
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<th>0% O$_2$-5% CO$_2$</th>
<th>21% O$_2$-5% CO$_2$</th>
<th>0% O$_2$-10% CO$_2$</th>
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</tr>
<tr>
<td>Cells/dish, $\times 10^6$</td>
<td>0.47 $\pm$ 0.08</td>
<td>0.55 $\pm$ 0.04</td>
<td>0.39 $\pm$ 0.11</td>
<td>0.40 $\pm$ 0.12</td>
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<tr>
<td>$^{51}$Cr release, %</td>
<td>9.9 $\pm$ 0.7</td>
<td>10.6 $\pm$ 0.5</td>
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<td>9.9 $\pm$ 0.3</td>
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Values for cells/dish and $^{51}$Cr release are means $\pm$ SE; n is no. of cultures. Confluent cultures of bovine aortic or main pulmonary artery endothelial cells in 35-mm dishes were incubated for 4 h at 37°C in minimal essential medium plus 12% fetal calf serum in atmospheres with varying gas tension: 0% O$_2$-5% CO$_2$, 0% O$_2$-10% CO$_2$, 21% O$_2$-5% CO$_2$, or 21% O$_2$-10% CO$_2$. Monolayers were washed 3 times with phosphate-buffered saline and were prepared for iodination using soluble lactoperoxidase (not conjugated to latex beads).

To study further the mechanism of effects of CO$_2$ on apical cell surface proteins, we incubated monolayers in varying gas tensions for 4 h in the presence of 1 µM cycloheximide. We found no effect of cycloheximide on changes in iodination of the 27-, 47-, and 52-kDa bands (data not shown). In a further attempt to assess the role of protein synthesis on the effects of hypercarbia, we...
incubated monolayers in varying gas tensions plus \[^{35}\text{S}\]methionine to label any newly synthesized proteins during the 4-h incubation period. Figure 4 shows that hypercarbia did not change \[^{35}\text{S}\]methionine incorporation into either cell lysates (A) or media (B). These results suggest that the 27-, 47-, and 52-kDa bands were not newly synthesized surface proteins and raise the interesting possibility that hypercarbia changes the apical cell surface display of proteins on endothelial cells.

Because carbonic anhydrase IV from bovine lung has been reported to have electrophoretic mobility consistent with a molecular mass of 52 kDa (25), we wondered whether hypercarbia increased BPAEC carbonic anhydrase IV expression. Using polyclonal antibodies to bovine and rat lung carbonic anhydrase IV, we were not able to demonstrate a consistent effect of hypercarbia on bovine main pulmonary artery carbonic anhydrase IV using ECL-Western immunoblots (data not shown).

**DISCUSSION**

These experiments demonstrate that 4 h of exposure of confluent cultures of endothelial cells to moderate hypercarbia, but not to acidosis, caused the appearance of three surface proteins of molecular masses of 27, 47, and 52 kDa. The effects of hypercarbia were only observed using iodination with lactoperoxidase conjugated to latex beads, suggesting that these substances are likely localized to the apical surface of the cells. Because cycloheximide did not inhibit this effect and because the bands were not labeled using \[^{35}\text{S}\]methionine, it is unlikely that they represented newly synthesized proteins.

**Fig. 3.** Effect of varying medium pH on iodination using lactoperoxidase conjugated to beads. Confluent cultures of BAEC in 35-mm dishes were incubated for 4 h at 37°C in MEM plus 12% FCS in 21% O\(_2\)-5% CO\(_2\) in medium that had been pH adjusted to 7.30 or 7.10 before incubation. Monolayers were washed 3 times with phosphate-buffered saline and were prepared for iodination using lactoperoxidase conjugated to latex beads. Arrows, bands at 46 and 190 kDa that were decreased in density by exposure to media of pH 7.10.

**Fig. 4.** Effect of varying gas tensions on radiolabeled proteins in cell extracts (A) and medium proteins (B). Confluent cultures of BAEC in 35-mm dishes were incubated for 4 h at 37°C in methionine-free MEM plus \[^{35}\text{S}\]methionine in atmospheres of either 21% O\(_2\)-5% CO\(_2\) or 21% O\(_2\)-10% CO\(_2\). Monolayers were washed 3 times with phosphate-buffered saline. At the end of the incubation period, media were collected, and monolayers were washed 3 times. Protein was precipitated from media with 10% trichloroacetic acid. Cells were extracted with 30 mM n-octyl glucoside. Aliquots of detergent extract supernatants (A) and solubilized media proteins (B) were separated using SDS-polyacrylamide gel electrophoresis.

These results suggest that carbonic anhydrase IV is not among the cell surface iodinated substances and increased in density after incubation in 10% CO\(_2\).
The effects of hypoxia on endothelial cell proteins have been extensively studied. Zimmerman et al. (30) and Graven et al. (7) have reported upregulation of proteins of molecular masses of 34, 36, 47, and 56 kDa (“hypoxia-associated proteins”) in endothelial cells of various origins. Prasada et al. (18) found that hypoxia induced synthesis of tropomyosin in porcine pulmonary artery endothelial cells. Kourembanas et al. (11, 12) have reported that hypoxia regulates expression of platelet-derived growth factor B and endothelin expression. Among potential membrane-associated proteins, Loike et al. (13) reported that hypoxia increased glucose transporter expression, King et al. (10) reported increased angiotensin-converting enzyme synthesis, and Weinhouse et al. (27) reported increased glycoprotein expression. We found that 4 h of exposure to moderate hypoxia modestly enhanced cell surface protein display as assessed by bead-immobilized lactoperoxidase-glucose oxidase.

Changes in environmental gas tensions also may change lung endothelial cell proteins in vivo. De La Fuente et al. (3) reported that exposure of rats to 48–60 h of hypoxia changed the pattern of display of lung membrane proteins accessible to a single transit of an intravascular extracellular reagent. These authors suggested that hypoxia altered luminal endothelial cell protein surface display.

Our results show that hypercarbia increased iodination of surface substances labeled with lactoperoxidase conjugated to latex beads but not with soluble lactoperoxidase. We interpret these results as showing selective effects of hypercarbia on the apical endothelial cell surface. Muller and Gimbrone (15) elegantly demonstrated surface selective iodination of confluent cultures of BAEC and human umbilical vein endothelial cells using lactoperoxidase conjugated to latex beads. In those experiments, the beads did not gain access to the basal (dish) surface of confluent endothelial monolayers, indicating that lactoperoxidase conjugated to beads selectively labeled the apical cell surface in adherent monolayers (15). Using a similar approach with soluble lactoperoxidase and lactoperoxidase conjugated to latex beads, Belloni and Nicolson (1) demonstrated that iodinated protein profiles from in situ labeled microvessels from a variety of organs, including lung, were similar to profiles derived from cultured BAEC. Hypercarbia-induced changes in cell surface substances were similar in our studies of BPAEC and BAEC. In contrast, Weinhouse et al. (27) reported differences between cultured BPAEC and BAEC in the effects of 4 h of hypoxia on glycoprotein expression (27).

Although cultured endothelial cells do not appear to be polarized by morphological evaluation, they are polarized in expression of some cell surface markers. As noted above, Muller and Gimbrone (15) demonstrated differences between apical and basal cell surface membrane proteins using the lactoperoxidase-latex bead technique. In addition, Narahara et al. (16) reported tissue factor expression to be highly polarized to the apical cell surface of human umbilical vein endothelial cells. Furthermore, Unemori et al. (25) and Sporn et al. (22) reported vectorial secretion of proteins by cultured endothelial cells to either apical or basal cell surfaces, which was dependent on the substance and/or mechanism of secretion. Surface polarization has also been reported for endothelial cells in vivo (20), but care must be taken in interpretation of these studies because of potential problems with adequate access of macromolecular or charged markers to the abluminal cell surface (19).

Hypoxia has been reported to change leukocyte adhesion to cultured vascular endothelial cells by endothelial cell surface changes (6). We are not aware of studies that demonstrate physiological changes in vectorial secretion or surface marker display by endothelial cells, with the exception of the effects of hypoxia, as described by De La Fuente et al. (3). However, in other polarized cells, disease-associated changes in apical and basal surface markers have been described (4, 26). For example, ischemia increased apical membrane display of Na-K pump in renal tubular epithelial cells, resulting in disrupted vectorial Na flux (4).

Bovine lung carbonic anhydrase IV has a molecular mass of 52 kDa and is an integral membrane protein (26). Because carbonic anhydrase IV has been localized to pulmonary microvascular endothelial cells in rat lungs (5) and to the apical cell membrane of cultured BPAEC (21), we assessed the effects of hypercarbia on carbonic anhydrase IV expression by pulmonary artery endothelial cells. Using Western immunoblots, we were not able to discern hypercarbia-induced changes in carbonic anhydrase IV expression in detergent extracts of BPAEC. However, further studies of apical surface expression are necessary to exclude this possibility completely.

We do not yet know the identity of the 27-, 47-, and 52-kDa proteins that were enhanced by exposure to hypercarbia. Hypercarbia causes increased prostaglandin release (9) and intracellular Ca²⁺ concentrations in endothelial cells (29). Both of these effects appeared related to decreased intracellular pH caused by hypercarbia (9, 29). Because intracellular pH may be a determinant of polarized protein secretion by some cells (2), we assessed the effects of changes in pH of media on endothelial cell surface iodination. We did not observe increases in the 27-, 47-, and 52-kDa substances under these conditions, suggesting that these effects of hypercarbia were not mediated by changes in pH. However, we did observe that acidosis decreased labeling of other bands.

In summary, we have found that hypercarbia changes the pattern of iodinated detergent-extractable substances associated with endothelial cells. We speculate that these changes are due to increased apical cell surface display of membrane-associated proteins. The identity of these substances awaits further study. These findings indicate that hypercarbia, like hypoxia, has the potential for changing endothelial cell physiology. Thus chronic hypercarbia may have other effects on the intact organism in addition to alteration of acid-base status.
REFERENCES


