Reduction in PO_2 Decreases the Fibrinolytic Potential of Cultured Bovine Endothelial Cells Derived From Pulmonary Arteries and Lung Microvasculature

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The effect of anoxia on the fibrinolytic potential of cultured endothelial cells derived from bovine pulmonary artery and bovine lung microvasculature was studied. Both cell types reacted with an increase in plasminogen activator inhibitor (PAI) activity and a decrease in the plasminogen activator (PA) activity in the media after incubation under anoxic conditions. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis followed by fibrin autography and reverse fibrin autography indicated that the change in fibrinolytic potential was due to an impaired release of PA and not an increase in the production of PAI. Although anoxia did not affect the viability of the cells as judged by ^{51}Cr release, their metabolism was influenced, which is reflected by increases in the levels of lactate in cell lysates and media. Furthermore, the effect of short-term anoxia on PA and PAI could not be reversed by reoxygenation for 24 hours. The results are discussed in terms of helping to explain the tendency of reocclusion after successful thrombolytic therapy, the development of pulmonary hypertension, and the thrombotic tendency of areas with an impaired circulatory supply.

THE FIBRINOLYTIC potential represented by plasminogen activators (PA) and PA inhibitors (PAI) of cultured endothelial cells is influenced by several mediators, with PAI seemingly more regulated than is PA. One condition thought to influence fibrinolytic activity in vivo is hypoxia. It has been shown that PA activity in rats decreases after severe short-term hypoxia whereas long-term hypoxia results in an increase.[1] PA release was also stimulated in an isolated pig ear preparation perfused with hypoxic solutions.[2] In humans, however, short-term acute hypoxemia did not increase blood fibrinolytic activity.[3] At present, data on the effects of hypoxia are rather inconsistent. In addition, there is little information available concerning the relationship between oxygen tension and PA/PAI activities of endothelial cells in culture. Because endothelial cells of the lung can be subjected to large changes in oxygen tension and because differences in the fibrinolytic patterns of endothelial cells from microvascular and macrovascular origins have been reported,[4] it was the aim of the present study to investigate the influence of reduced oxygen tension on PA and PAI activities in cultured endothelial cells isolated from both large and small vessels of the lung.

MATERIALS AND METHODS

Nu-Serum (Collaborative Research, Waltham, MA); Dulbecco's modified essential medium (DMEM), fetal bovine serum (FBS), thrombin, type IV collagenase, lactate determination kit, bovine serum albumin (BSA) (Sigma Chemical Co, St Louis); bovine plasminogen containing fibrinogen (Poviet, Oss, The Netherlands); H-D-Nle-HHT-Lys-pNA (Spectrozyme plasmin), goat anti–tissue-type PA (anti–t-PA) antibodies, goat anti–urokinase PA (anti–u-PA) antibodies, affinity-purified human high–molecular weight (mol wt) urokinase (American Diagnostica, New York); and low–mol wt protein standards for sodium dodecyl sulfate (SDS) gel electrophoresis, acrylamide, bisacrylamide, and SDS (BioRad Laboratories, Richmond, CA) were obtained as indicated.

Isolation and cultivation of endothelial cells. Bovine pulmonary artery endothelial cells (BPAEC) were isolated and characterized as described previously for calf aorta.[5] Bovine lung microvascular endothelial cells (BLMEC) were obtained by collagenase treatment (1 mg/mL) of peripheral lung tissue for 20 minutes at 37°C. The suspension was centrifuged and the pellet resuspended and seeded into Petri dishes. Individual clones, identified by their endothelial-like morphology, were picked, seeded into new Petri dishes, grown to confluency, and characterized as endothelium on the basis of morphology, acetylated low-density lipoprotein uptake, von Willebrand factor VIII staining, and angiotensin-converting enzyme activity.[5] BPAEC and BLMEC were cultured in medium containing DMEM plus 5% Nu-serum and 5% FBS. All endothelial cells used for the experiments were between the fifth and seventh passage.

Incubation of endothelial cells under normoxic and anoxic conditions. BPAEC and BLMEC, respectively, were seeded into 24-well cluster plates (GIBCO, Grand Island, NY) at 5×10^4 cells/well. At confluency the monolayers were washed with Hanks' balanced salt solution bufferd with HEPES (10 mmol/L), pH 7.4, and 0.5 mL of DMEM plus 0.5% BSA were added to each well. The plates were transferred to modular incubator chambers (Vangard International, Inc, Neptune, NJ) and flushed with 95% N_2 and 5% CO_2 to simulate anoxic conditions or 21% O_2, 74% N_2, and 5% CO_2 for normoxic controls. Cells were incubated for 8, 24, and 48 hours. After each incubation period, the media were harvested, centrifuged at 300 g, and stored at −70°C. Cell lysates were prepared by incubating the remaining monolayers with 0.25% Triton X-100 in phosphate-buffered saline for 15 minutes at 37°C. The lysates were also centrifuged at 300 g and the supernatants stored at −70°C.

Characterization of fibrinolytic activity produced by BPAEC and BLMEC. SDS gel electrophoresis[6] was carried out on the conditioned media and lysates of BPAEC and BLMEC. The gels were then used for detection of PA and PAI activities by a plasminogen-rich fibrin overlay technique[7] and reverse fibrin autography.[8]

Determination of PA and PAI activity. PA activity was measured by using a slight modification of a previously described titration assay.[9] Human high–mol wt urokinase (u-PA), calibrated against the international standard preparation for u-PA (Institute for Biological Standards and Control, Holly Hill, Great Britain), was substituted for t-PA, and the fibrin degradation products, which

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Submitted October 13, 1987; accepted February 2, 1988.

Supported by Grants HL-36526 and HL-01775 from the National Institutes of Health.

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0006-4971/88/7106-0033$3.00/0

can act as stimulators, were omitted. To measure PAI activity, a functional assay using immobilized monoclonal antibodies against t-PA[10] was used with the following modifications: a monoclonal antibody against u-PA (MPW5UK)[11] was used to immobilize human high-mol wt urokinase (which served as the PA), and the fibrinogen fragments originally used as stimulators were omitted.

Determination of the viability of the cultured endothelial cells. To determine the effect of anoxia on viability, cells prelabeled with ^{51}Cr (1 μC/well for two hours at 37°C) were treated as described earlier. After 48 hours' incubation under normoxic or anoxic conditions, amounts of ^{51}Cr released into the media and ^{51}Cr bound to the cells were determined by scintillation counting. Viability was represented as the percentage of total ^{51}Cr found in both the cells and media that was present in the media after the various incubations.

Determination of lactate levels. Lactate levels in conditioned media and cell lysates were measured at the various time points by using a kit that spectrophotometrically monitors the reduction of nicotinamide adenine dinucleotide (NAD) to NADH (NAD, reduced form) as a result of the conversion of lactate to pyruvate by lactate dehydrogenase.

Statistical analysis. Data were compared statistically by using Student's *t* test for paired analysis. The *P* values calculated in all Figures and Tables are based on differences between anoxic values and the corresponding control (normoxic) values.

RESULTS

Characterization of fibrinolytic components in BPAEC and BLMEC. The fibrinolytic activity elaborated by BPAEC and BLMEC, under control conditions as revealed by fibrin autography and reverse fibrin autography, produced two lysis zones corresponding to mol wts of 110 and 45 kd (Fig 1). The lysis zone at 45 kd could partially be quenched by the monoclonal anti-u-PA antibody (MPW5UK)[11] as well as by the polyclonal anti-u-PA antibodies. The 110-kd lysis zone was fully depleted by the anti-t-PA monoclonal antibody (MPW3VPA)[12] and the polyclonal anti-t-PA antibodies. Anti-t-PA antibodies had no effect on the 45-kd lysis zone, and anti-u-PA antibodies had no effect on the 110-kd lysis zone. From this it was concluded that the 45-kd lysis zone represented u-PA, the predominant PA released by BPAEC and BLMEC, and that t-PA was present in a complexed form, presumably with a PAI, as indicated by the lysis zone at 110 kd. A 52-kd lysis-resistant zone revealed by reverse fibrin autography was also visualized and indicated free PAI.

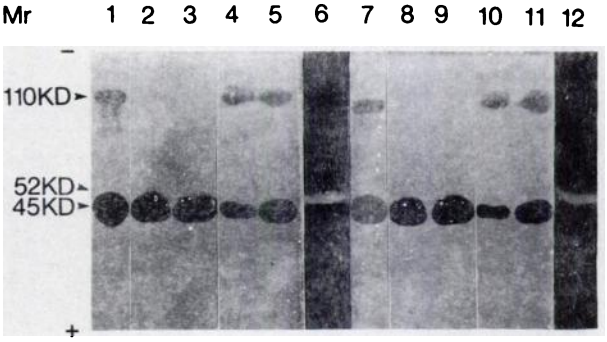

Fig 1. SDS-polyacrylamide gel electrophoresis (PAGE) of 24-hour conditioned media (CM) of BPAEC and BLMEC followed by fibrin autography (lanes 1 to 5 and 7 to 11, respectively) or reverse fibrin autography (lanes 6 and 12). Untreated CM of BPAEC (lanes 1 and 6) and BLMEC (lanes 7 and 12), CM of BPAEC and BLMEC preincubated with monoclonal anti-t-PA antibody MPW3VPA (lanes 2 and 8), with polyclonal anti-t-PA antibody (lanes 3 and 9), with monoclonal anti-u-PA antibody MPW5UK (lanes 4 and 10), and with polyclonal anti-u-PA antibody (lanes 5 and 11).

Effect of anoxia on PA and PAI activity. As can be seen in Figs 2 and 3, PAI activity in the conditioned media of both BPAEC and BLMEC increased under anoxic conditions with a concomitant decrease in PA activity. Under normoxic conditions, however, PAI as well as PA activity in the conditioned media of both cell types increased over time, ie, the increase in PAI activity was not accompanied by a decrease in PA activity. The respective insets in Figs 2 and 3 show a time-dependent increase in PAI activity in cell lysates during the anoxic incubation. No PA activity in cell lysates could be detected.

Although BPAEC and BLMEC responded similarly to anoxia, under normoxia significantly less PAI activity was found in the cell lysates of BPAEC as compared with BLMEC (3.86 ± 0.59 *v* 5.98 ± 0.16, $P < .01$) and significantly more PA activity in the conditioned media of BLMEC as compared with BPAEC (2.60 ± 0.17 *v* 1.85 ± 0.59, $P \leq .05$).

To investigate whether the effect of anoxia is reversible, BPAEC and BLMEC were incubated for 2, 8, and 24 hours under normoxic or anoxic conditions. Thereafter, the monolayers were washed, new media added, and incubation continued for an additional 24 hours under normoxia. Data given in Table 1 indicate that the effect of anoxia on PAI and PA activities was not reversible, at least not within 24 hours after anoxia.

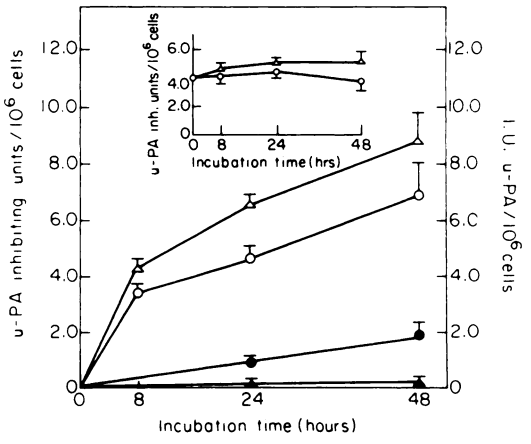

Fig 2. Effect of anoxia on PA and PAI activity of BPAEC. PA activity in conditioned media after anoxic (closed triangles) or normoxic (closed circles) incubation is given in international units of u-PA. PAI activity in conditioned media and cell lysates (inset) after anoxic (open triangles) or normoxic (open circles) incubation is given in u-PA-inhibiting units. Results represent mean values of three experiments, each performed in triplicate with two different clones from two different animals. With the exception of the eight hours' values for the cell lysates (inset), which were not significantly different from the respective controls, all values obtained with cells kept under anoxia were significantly different from those obtained with the respective normoxic controls ($P < .05$).

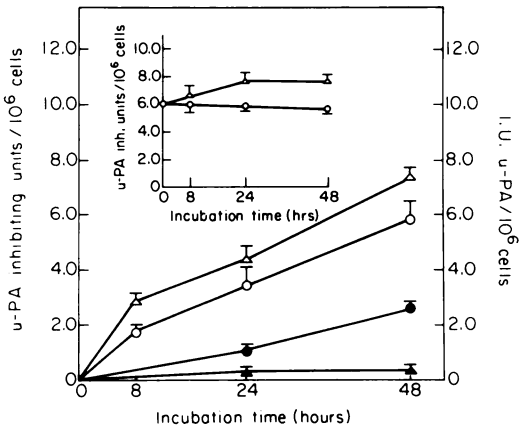

Fig 3. Effect of anoxia on PA and PAI activity of BLMEC. PA activity in conditioned media after anoxic (closed triangles) or normoxic (closed circles) incubation is given in international units of u-PA. PAI activity in conditioned media and cell lysates (inset) after anoxia (open triangles) or normoxia (open circles) is given in u-PA–inhibiting units. Results represent mean values of three experiments, each performed in triplicate with two different clones from two different animals. With the exception of the eight hours' values for the cell lysate (inset), which were not significantly different from the respective controls, all values obtained with cells kept under anoxia were significantly different from those obtained with the respective normoxic controls ($P < .05$).

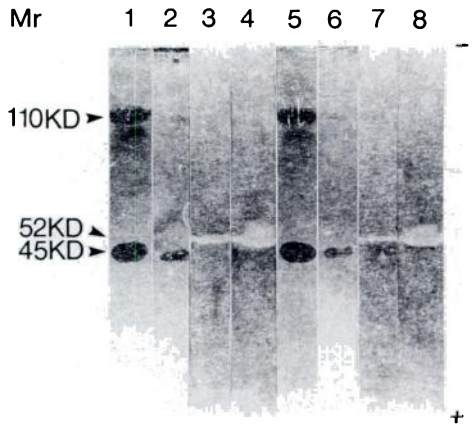

Fig 4. SDS-PAGE of conditioned media (CM) of BPAEC or BLMEC kept for 48 hours under normoxic or anoxic conditions followed by fibrin autography (lanes 1, 2, 5, and 6) or reverse fibrin autography (lanes 3, 4, 7, and 8). CM of BPAEC after 48 hours' normoxia, lanes 1 and 3; CM of BPAEC after 48 hours' anoxia, lanes 2 and 4; CM of BLMEC after 48 hours' normoxia, lanes 5 and 7; and CM of BLMEC after 48 hours' anoxia, lanes 6 and 8.

Because the reduction in PA activity could be due to either a decrease in PA production or an increase in PAI, conditioned media from BPAEC and BLMEC kept for 48 hours under normoxic or anoxic conditions were subjected to SDS gel electrophoresis followed by fibrin autography or reverse fibrin autography. Figure 4 clearly shows that as a result of anoxia the lysis zones at 45 and 110 kd were markedly reduced whereas the lysis-resistant zone at 52 kd was increased. The results indicate a decrease in PA production that subsequently results in an increase in the amount of free PAI. When conditioned media from cells incubated under normoxia for 8 and 24 hours, respectively, were applied to fibrin autography and reverse fibrin autography and when lysis zones and lysis-resistant zones were compared with those obtained with conditioned media from cells kept for 48 hours under normoxia, the respective lysis zones and lysis-resistant zones were smallest for the eight-hour, intermediate for the 24-hour, and greatest for the 48-hour conditioned media. When the same experiment was performed with conditioned media obtained from cells incubated under anoxia for the time points described before, the respective lysis zones were smaller, and the respective lysis-resistant zones were greater when compared with the normoxic control. The lysis-resistant zones, however, increased in size over time, whereas the respective lysis zones did not (data not shown).

Effect of anoxia on cell viability. ^{51}Cr release assays indicate that there was no significant effect of anoxia on the viability of either BPAEC or BLMEC. The mean values for ^{51}Cr release after 48 hours' anoxia and normoxia were 30.5% ± 0.9% and 30.2% ± 1.8%, respectively, for BPAEC and 37.9% ± 0.9% and 39.3% ± 0.7%, respectively, for BLMEC. Anoxia did, however, affect cell metabolism (Table 2). Lactate levels were increased in the media as well as in the lysates of both cells incubated under anoxic conditions.

Table 1. PAI and PA Activity of BPAEC and BLMEC

	Hours of AO + 24 h of NO			
	2	8	24	C
BPAEC CM PAI	6.4 ± 0.17*	6.4 ± 0.15*	7.0 ± 0.11*	5.4 ± 0.57
PA	1.4 ± 0.20	1.0 ± 0.23*	<0.05*	1.7 ± 0.65
CL PAI	4.9 ± 0.16*	5.1 ± 0.17*	6.2 ± 0.23*	4.5 ± 0.17
BLMEC CM PAI	3.6 ± 0.05	3.7 ± 0.06*	4.1 ± 0.15*	3.3 ± 0.20
PA	0.9 ± 0.11	0.7 ± 0.05*	<0.05*	1.0 ± 0.20
CL PAI	6.1 ± 0.20	6.2 ± 0.15	7.1 ± 0.20*	6.0 ± 0.26

PAI activity (u-PA–inhibiting u/10^6 cells) and PA activity (IU u-PA/10^6 cells) were determined in conditioned media and lysates of BPAEC and BLMEC preincubated for 2, 8, and 24 hours under anoxic conditions followed by 24 hours under normoxic conditions and BPAEC and BLMEC incubated for the same time periods under normoxic conditions.

Data are the mean values of three experiments, each performed in triplicate with two different clones obtained from two different animals. Values are given as means ± SD.

Abbreviations: C, control; AO, anoxic conditions; NO, normoxic conditions; CL, conditioned lysates.

*$P < .05$.

Table 2. Lactate Levels of BPAEC and BLMEC

	24 h		48 h	
	NO	AO	NO	AO
BPAEC CM	0.37 ± 0.02	0.50 ± 0.06*	0.60 ± 0.04	1.00 ± 0.01*
CL	18.50 ± 1.90	21.40 ± 2.40*	17.30 ± 1.70	20.20 ± 1.90*
BLMEC CM	0.42 ± 0.03	0.59 ± 0.02*	0.74 ± 0.03	0.92 ± 0.02*
CL	18.40 ± 2.30	24.90 ± 1.70*	17.50 ± 1.10	25.40 ± 4.60*

Lactate levels in conditioned media (mg/10^6 cells) and lysates (μg/10^6 cells) of BPAEC and BLMEC kept for 24 and 48 hours were determined under normoxic or anoxic conditions. The results are the mean values of three experiments, each performed in triplicate with two different clones from two different animals. Values are given as means ± SD.

*$P < .05$.

DISCUSSION

The results indicate that an alteration in oxygen tension affects the fibrinolytic capacity of cultured endothelial cells. Both large- and small-vessel endothelial cultures respond in a similar way to the reduction in oxygen tension: with a decrease in PA activity and an increase in PAI activity as compared with normoxic controls. The time-dependent increase in PAI activity under normoxia, as demonstrated by functional assays as well as by autographic techniques was not, however, accompanied by a decrease in PA activity as was the case with anoxia. Our results indicate that the decreased fibrinolytic activity is due to an impaired release of PA and not an increase in the release of PAI, as clearly revealed by the reduced lysis zones in the fibrin autography of the conditioned media. This diminution of PA was not due to anoxia-mediated cell death because cell viability was not significantly affected. Anoxia did affect cell metabolism as shown by the significant increase in lactate levels and by the irreversibility of fibrinolytic capacity after 24 hours' incubation under normoxic conditions. PA activity decreased after a two-hour anoxic period and continued to do so as long as anoxia was maintained. The data indicate that reduction of oxygen tension caused the cells to obtain their energy requirements through anaerobic metabolism, which in turn induced a time-dependent reduction in PA secretion and an overall impaired fibrinolytic activity.

In conclusion, our data suggest that anoxic conditions may contribute to the tendency of reocclusion after successfully lysed thrombotic occlusions. This would occur because the endothelial cells downstream of the occluded area would have an impaired fibrinolytic potential that would remain so for some time, even after restoration of an adequate oxygen supply. Furthermore, this mechanism may also play a role in the deposition of fibrin in the pulmonary circulation after a reduction in ambient oxygen tension, eg, because of a high altitude. Such fibrin deposits could in turn lead to the development of pulmonary hypertension. The tendency toward thrombosis in areas with impaired circulation might also be in part a result of the effect of reduced oxygen tension on the fibrinolytic potential of endothelial cells.

REFERENCES

1. Risberg B, Stenberg B: Modulation of tissue fibrinolysis from hypoxia and hyperoxia. Thromb Res 38:129, 1985

2. Tappy L, Hauert J, Bachmann F: Effects of hypoxia and acidosis on vascular plasminogen activator release in the pig ear perfusion system. Thromb Res 33:117, 1984

3. Stegnar M, Peternel P, Chen JP: Acute hypoxemia does not increase fibrinolytic activity in man. Thromb Res 45:333, 1987

4. Speiser W, Anders E, Preissner KT, Wagner O, Mueller-Berghaus G: Differences in the coagulant and fibrinolytic activities of cultured human endothelial cells derived from omental tissue microvessels and umbilical veins. Blood 69:964, 1987

5. Hoover RL, Karnovsky MJ, Austen KF, Corey EJ, Lewis RA: Leukotriene B_4 action on endothelium mediates augmented neutrophil/endothelial adhesion. Proc Nat Acad Sci USA 81:2191, 1984

6. Laemmli UK: Cleavage of structural proteins during the assembly of the head of the bacteriophage T_4. Nature 227:680, 1970

7. Granelli-Piperno A, Reich E: A study of proteases and protease-inhibitor complexes in biological fluids. J Exp Med 148:223, 1978

8. Erickson LA, Lawrence DA, Loskutoff DJ: Reverse fibrin autography: A method to detect and partially characterize protease inhibitors after sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Anal Biochem 137:454, 1984

9. Korninger C, Wagner O, Binder BR: Tissue plasminogen activator inhibitor in human plasma: Development of a functional assay system and demonstration of a correlating M_r = 50,000 antiactivator. J Lab Clin Med 105:718, 1985

10. Speiser W, Wojta J, Korninger C, Kirchheimer JC, Zazgornik J, Binder BR: Enhanced fibrinolysis caused by tissue plasminogen activator release in hemodialysis. Kidney Int 32:280, 1987

11. Wojta J, Kirchheimer JC, Turcu L, Christ G, Binder BR: Monoclonal antibodies against human high molecular weight urinary urokinase. Application for affinity purification of urinary prourokinase. Thromb Haemost 55:347, 1986

12. Wojta J, Turcu L, Wagner O, Korninger C, Binder BR: Evaluation of fibrinolytic capacity by a combined assay system for t-PA antigen and function using monoclonal anti t-PA antibodies. J Lab Clin Med 109:665, 1987