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Hypoxia induces endothelial cell synthesis of membrane-associated proteins

(hypoxia/endothelium/coagulation)

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ABSTRACT **Hypoxemia is associated with a prothrombotic tendency. In this study we report the purification and partial characterization of an activator of a central coagulation component, factor X, induced in endothelium by exposure to hypoxia (hypoxia-induced factor X activator or X_{act}). Expression of X_{act} occurred in a reversible manner when endothelial cell cultures were exposed to hypoxia or sodium azide but not in response to a variety of other alterations in the cellular milieu, such as heat shock or glucose deprivation. The activity of X_{act}, which was not detected in normoxic endothelial cells, was maximal under acidic conditions, pH 6.0–6.8, which often coexist with hypoxia in an ischemic milieu. By sequential isoelectric focusing and preparative SDS/PAGE of endothelial membrane-rich fractions, X_{act} was purified ≈19,000-fold and found to be a single-chain, ≈100-kDa polypeptide with pI ≈5.0. Activation of factor X by purified X_{act} was not affected by blocking antibodies to other coagulation proteins or by phenylmethylsulfonyl fluoride or leupeptin but was prevented by mercury chloride or iodoacetamide. In addition to the induction of X_{act}, two-dimensional gel analysis of membrane fractions from metabolically labeled hypoxic endothelial cultures revealed two groups of ≈10 additional spots: (*i*) a group for which expression was maximal after 24 hr and (*ii*) a group for which expression continued to increase up to 48 hr. The pattern of hypoxia-mediated modulation of protein expression was distinct from that seen with other cellular stimuli but could be duplicated, in part, by sodium azide. These results indicate that hypoxia elicits a specific biosynthetic response, including the expression of endothelial cell-surface molecules that can alter cellular function and may potentially serve as markers of hypoxemic vessel-wall injury.**

Exposure of endothelium to environments of low oxygen tension is a frequent occurrence in various disorders, especially those associated with compromise of the circulation. Two crucial functions of endothelium, maintenance of a permeability barrier and preservation of the fluidity of blood, are adversely affected by levels of hypoxia that occur in ischemic syndromes (1, 2). Based on studies in cell culture, such hypoxia-mediated perturbation of endothelial cell (EC) functions results from alterations in metabolic pathways, not from a change in viability with death of the cell monolayer (3, 4). For example, exposure of bovine aortic ECs to hypoxia led to an increase in their permeability to solutes, but these changes were reversible on restoration of cultures to normoxia (4).

Hypoxemia has long been associated with a prothrombotic tendency, especially in the setting of deep vein thrombosis, where stasis of an extremity in an animal model results in extreme local hypoxemia and fibrin deposition on the cusps of vein valves (2). In a previous study (4), we showed that hypoxic cultured ECs expressed an apparently unusual procoagulant activity, allowing them to activate directly a central coagulation component, factor X. We now report further characterization of the hypoxia-induced factor X activator (X_{act}) and the identification of a group of EC surface-associated proteins, the expression of which is induced/enhanced by hypoxia (termed oxygen-regulated proteins or ORPs) (5, 6).

METHODS

Induction of Hypoxia. Bovine aortic ECs were grown and rendered hypoxic, as described (4) (smooth muscle cells were obtained from the same calf aortas by further scraping vessels after removal of ECs). During these experiments, the pO_2 of the culture medium was ≈14 mmHg (1 mmHg = 133 Pa), and the pH of the medium remained constant (when Hepes was omitted from the medium, pH fell). By 72 hr of EC incubation under hypoxic conditions, glucose concentration in the medium fell from 5 to 2.5 mM. This degree of glucose depletion did not affect X_{act} expression and did not induce synthesis of any of the proteins seen when cultures were labeled in glucose-free medium (see below). ATP levels were determined by using the luciferase assay (7), and protein synthesis was assessed by the incorporation of radiolabeled amino acids (8).

Activation of Factor X by Hypoxic ECs. Intact monolayers, cell suspensions, membrane-rich fractions, or samples from the purification procedure described below were incubated with purified bovine factor X (1 μM or as stated) at 37°C for the indicated time, and then the presence of factor Xa was assessed by chromogenic substrate, coagulant, or radiometric assays (4, 9, 10).

Purification of X_{act}. This purification included preparative isoelectric focusing and SDS/PAGE (11). ECs exposed to hypoxia for 48 hr (5×10^9 cells) were washed in buffer, scraped from the growth surface, and resuspended in Veronal buffer (20 mM; pH 7.8) containing phenylmethylsulfonyl fluoride (2 mM) to elute X_{act}. The eluate was diluted in 50 ml of 1.5% Ampholyte (pH range 3–10, Bio-Rad)/0.1% octyl β-glucoside, and isoelectric focusing (Rotofor Cell, Bio-Rad) was done. Fractions were dialyzed against 0.4 M Tris/HCl, pH 7.5/0.2 M NaCl/0.1% octyl β-glucoside, diluted in Veronal-buffered saline, and tested for X_{act} activity. Next, preparative nonreduced SDS/PAGE (10%) was done, and proteins in the gel were either visualized by silver staining or

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Abbreviations: ORP, oxygen-regulated protein; X_{act}, hypoxia-induced factor X activator; TNF, tumor necrosis factor/cachectin; EC, endothelial cell.

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electroeluted from the gel and assessed for X_{act} activity [the latter after SDS removal (12)].

Metabolic Labeling. EC metabolic labeling was done by incubating cultures in methionine-poor minimal essential medium/5% dialyzed fetal calf serum/[^{35}S]methionine at 0.2 mCi/ml (1 Ci = 37 GBq), the latter added 8 hr before the end of an experiment.

Two-Dimensional Gel Analysis. This was done after exposure of EC monolayers to the test conditions by suspending the cells in (10 mM Tris, pH 7.2)/aprotonin (at 100 units/ml), Dounce homogenizing, adding additional buffer (10 mM Tris/10 mM NaCl/10 mM KCl/5 mM $CaCl_2$/2 mM $MgCl_2$/0.5 M sucrose/aprotinin at 100 units/ml), and centrifuging at low speed to remove debris/nuclei. The supernatant was then ultracentrifuged (100,000 × *g* for 2 hr), and the membrane-rich pellet (from ≈10^6 cells) was solubilized in SDS/gel buffer and subjected to two-dimensional gel analysis (13). Gels were run by Protein DataBase (Huntington Station, NY) and analyzed with the PDQuest system (14).

RESULTS

Hypoxia-Induced, EC-Dependent Factor X Activation. EC monolayers grown to confluence in normoxia and then placed in hypoxia for 3 days maintained their viability: (*i*) production of ATP continued at levels of ≈75% that seen in normoxia (7), (*ii*) protein synthesis also continued with only a decrease by 20–30% in incorporation of radiolabeled amino acids into protein as compared with normoxia (4), (*iii*) uptake of the vital dye trypan blue was not increased (4), and (*iv*) after restitution to normoxia, the cells proliferated on further subculturing.

In contrast to this general maintenance of "housekeeping" functions necessary for cell viability, there were subtle alterations in properties of the hypoxic EC, which had implications for their central role in vascular homeostasis. Previously, we had observed that on exposure to hypoxia, ECs acquired the ability to shorten the clotting time of recalcified plasma, due to the expression of a cell-surface procoagulant activity that directly activated factor X (4). This hypoxia-induced X_{act} was not seen in intact cell monolayers or subcellular fractions of normoxic cultures, and its expression in hypoxic ECs was blocked by cycloheximide addition to culture medium, suggesting a role for *de novo* protein synthesis. Furthermore, mercuric chloride blocked factor X activation by hypoxic ECs, whereas phenylmethylsulfonyl fluoride had no effect, suggesting that a sulfhydryl group was necessary for X_{act} activity. In contrast to the effect of hypoxia, exposure of ECs to heat shock, glucose deprivation, or the cytokine tumor necrosis factor (TNF) did not induce an activity similar to X_{act} (data not shown). Furthermore studies with metabolic inhibitors showed that neither 2-deoxyglucose nor fluoride (which inhibit glycolysis) induced X_{act}, whereas sodium azide, an inhibitor of the electron-transport chain, did (data not shown). Activator expression was predominately on the cell surface, as experiments after freeze–thaw lysis of hypoxic ECs did not demonstrate additional X_{act} activity.

Our culture system was set up to achieve selective hypoxia (for example, pH did not vary beyond 7.3–7.6), but in the ischemic milieu hypoxemia is closely associated with acidosis. In this context, when ECs in medium without Hepes were maintained for 48 hr in hypoxia, pH of the medium fell to 6.5–6.8. This change led us to examine pH effect on factor X activation by hypoxic EC cultures. Although the pH optimum for activation of factor X by the extrinsic and intrinsic systems is at or about neutral pH, which is not surprising because neutral proteases are involved (15), X_{act} was more effective in a lower pH range (Fig. 1 *A–B*). For example, at pH 6.8, V_{max} for factor Xa formation was increased ≈4-fold

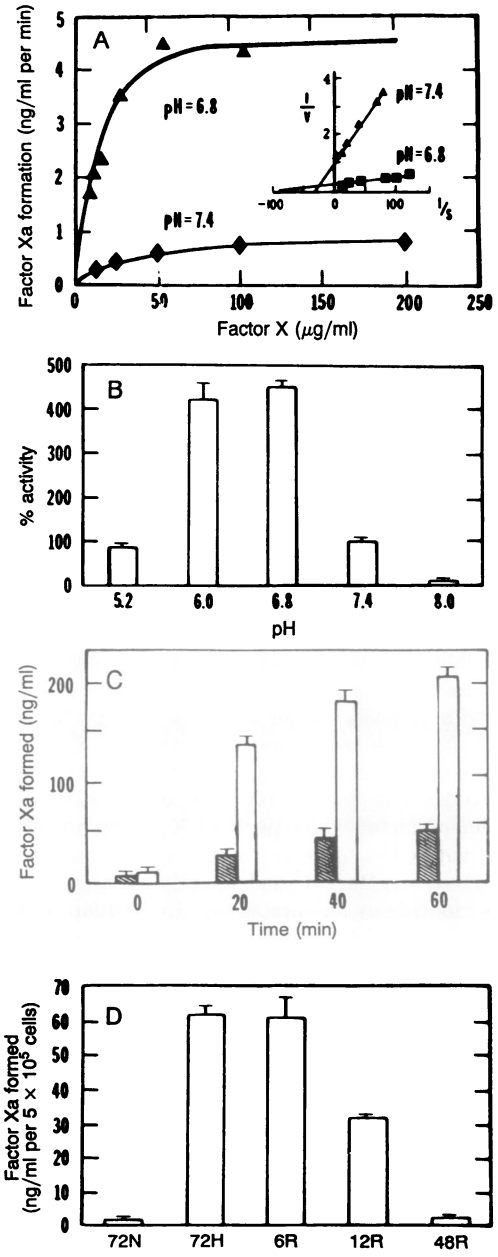

FIG. 1. Factor X activation by hypoxic endothelial cells: effect of pH and reversibility after exposure to ambient air. (*A*) EC monolayers grown in normoxia were incubated in hypoxia for 48 hr and incubated in buffer with pH 6.8 (▲) or 7.4 (◆) in the presence of factor X. Factor Xa formation is shown as ng/ml per min. (*Inset*) Double reciprocal plots. (*B*) EC monolayers were made hypoxic for 48 hr, incubated with 1 μM factor X at the indicated pH for 30 min at 37°C. Factor X activation is shown as a percentage of that seen at pH 7.4. (*C*) EC monolayers were incubated for 72 hr in hypoxia and resuspended in either plasma deficient in factors II/VII/X (open bars) or Veronal-buffered saline (hatched bars) supplemented with ^{3}H-labeled factor X (100 μg/ml). Then, samples were removed for the radiometric assay to detect factor Xa formation. (*D*) EC monolayers were incubated for 72 hr in hypoxia or normoxia and then exposed to ambient air, and factor Xa formation was studied after adding 1 μM factor X for 30 min at 37°C. 72N or 72H, normoxia or hypoxia for 72 hr, respectively; R, remainder, which are hypoxia for 72 hr followed by the indicated time in hr for normoxia (i.e., reoxygenation).

(5.1 versus 1.2 ng/ml per min) and K_m fell ≈2-fold (28 versus 13 μg/ml) compared with these parameters at pH 7.4 (Fig. 1*A*). Over a broader pH range, it was evident that the optimum pH for factor X activation was 6.0–6.8, whereas outside these pHs factor Xa formation decreased (Fig. 1*B*).

These data indicated that other environmental factors likely to occur during hypoxemia, such as acidosis, could modulate functional activity of X_{act}. In this context, experiments in which hypoxic ECs were incubated with ^{3}H-labeled factor X in the presence of plasma showed that factor Xa formation did occur in a complex, more physiologically relevant system (Fig. 1*C*). In fact, factor Xa formation by hypoxic ECs was more effective in the plasma-based system than in buffer with only factor X. Normoxic endothelial cells, under the same conditions, did not activate factor X (data not shown). Induction of X_{act} was reversible, as after exposure to normoxia, its activity diminished back to baseline (Fig. 1*D*).

Purification of X_{act} from Hypoxic Cultured ECs. To further assess the properties and potential functional significance of X_{act}, we undertook its purification (Fig. 2). Isoelectric focusing of membrane-rich fractions from hypoxic ECs demonstrated one peak of X_{act} activity (defined as factor X activation in the presence of only purified factor X that was not inhibited by antibody to tissue factor, factors IX or VIII) centered about pH ≈5.0 and separated from much of the protein (Fig. 2 *Upper*). Preparations from normoxic ECs did not demonstrate similar X_{act} activity. Nonreduced SDS/PAGE of membrane-rich preparations from hypoxic cultures demonstrated the expected complex pattern when stained for protein (Fig. 2 *Lower, A*), but gel elution showed only a single peak of X_{act} activity corresponding to ≈100 kDa (Fig. 2 *Lower, B*). The latter observation led us to combine isoelectric focusing, SDS/PAGE, and electroelution to prepare small amounts of purified X_{act} (Fig. 2 *Lower, C–E*). Extracts of membrane-rich fractions from ≈2 × 10^{10} hypoxic ECs were first subjected to preparative isoelectric focusing. Fractions with peak X_{act} activity, those corresponding to pH ≈5.0 in the gradient, were applied to preparative SDS/PAGE. The gel was then sliced, and pieces corresponding to ≈100 kDa were electroeluted, tested for X_{act} activity, and reapplied to analytical SDS/PAGE under nonreduced (Fig. 2 *Lower, C*) or reduced (Fig. 2 *Lower, E*) conditions. In each case, a single band of ≈100 kDa was seen. Gel elution of material identical to that in Fig. 2 *Lower, C* demonstrated a single peak of X_{act} activity only in the region of the gel corresponding to the protein band. In reduced gels, no X_{act} activity could be detected. With these steps, a purification of ≈19,000-fold was achieved, and 160 ng of homogeneous activator was recovered with a total yield of ≈12% (data not shown). The same procedure applied to membrane-rich fractions extracted from normoxic cultures resulted in no recovery of X_{act} activity. The similarity of purified X_{act} to the procoagulant activity observed in hypoxic ECs was apparent from the inhibition of X_{act} activity by iodoacetamide and mercury chloride but not by neutralizing antibodies to coagulation factors IX, VIII, or tissue factor (data not shown).

Hypoxia-Mediated Expression of EC Membrane-Associated ORPs. The observation that hypoxia induced the expression of X_{act} led us to examine the spectrum of membrane-associated proteins the expression of which was modulated in hypoxia by two-dimensional gel electrophoresis. With this approach, it was apparent that hypoxia modulated protein expression: certain spots/proteins were suppressed, the intensity of another group of spots was enhanced, and a third set of spots appeared to be induced *de novo*. The autoradiograms in Fig. 3 focus on the ORPs or pattern of spots the expression of which was induced or enhanced by hypoxia, compared with the normoxic control (Fig. 3*A*), based on time course of their appearance after initiation of hypoxic conditions. Two groups are evident: the first group consisted of ≈10 ORPs (Fig. 3*B*), which appeared within 24-hr exposure to hypoxia, and the second group consisted of an additional 10 ORPs, evident within 48 hr (Fig. 3*C*). Computer analysis of 20 experiments by the PDQuest system (14) confirmed the existence of these two groups of ORPs (Fig. 3 *DI–II*, termed early and late ORPs, respectively). Most ORPs depicted in Fig. 3*D* were induced, but careful study of early ORPs 1333, 3009, and 5418 and late ORP 8415 demonstrated that they resulted from enhanced expression of proteins that could be detected on gels from normoxic cultures.

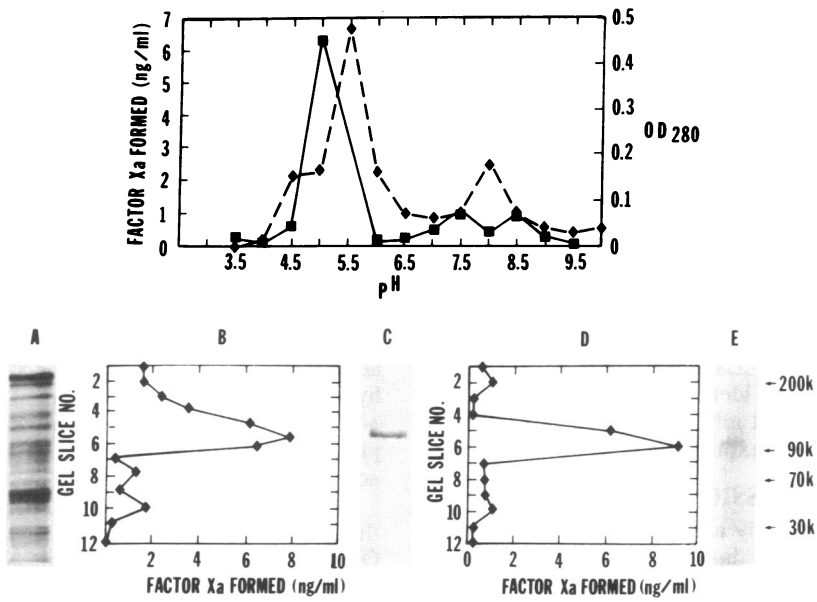

FIG. 2. Purification of hypoxia-induced endothelial factor X activator: isoelectric focusing, SDS/PAGE, and gel elution. (*Upper*) ECs (≈5 × 10^9) were made hypoxic for 48 hr, extracted with Veronal buffer and subjected to preparative isoelectric focusing. pH is plotted versus OD_{280} (– – –) and factor Xa formation (——). (*Lower*) Hypoxic EC extracts were subjected to SDS/10% PAGE, and lanes of the gel were then silver stained (*A*) or gel eluted and assayed for ability of samples to form factor Xa (*B*). (*C*) Hypoxic EC membrane extracts were subjected to isoelectric focusing, fractions with peak activity were then run on preparative SDS/10% PAGE, and slices of the gel were electroeluted. Material eluted from the slices with factor X-activating ability (slices 5 and 6) was pooled and rerun on nonreduced SDS/10% PAGE. Lanes of the latter gel were either silver-stained (*C*) or gel-eluted and assayed for ability of samples to form factor Xa (*D*). The gel-eluted material in lane D was also subjected to reduced SDS/PAGE (*E*).

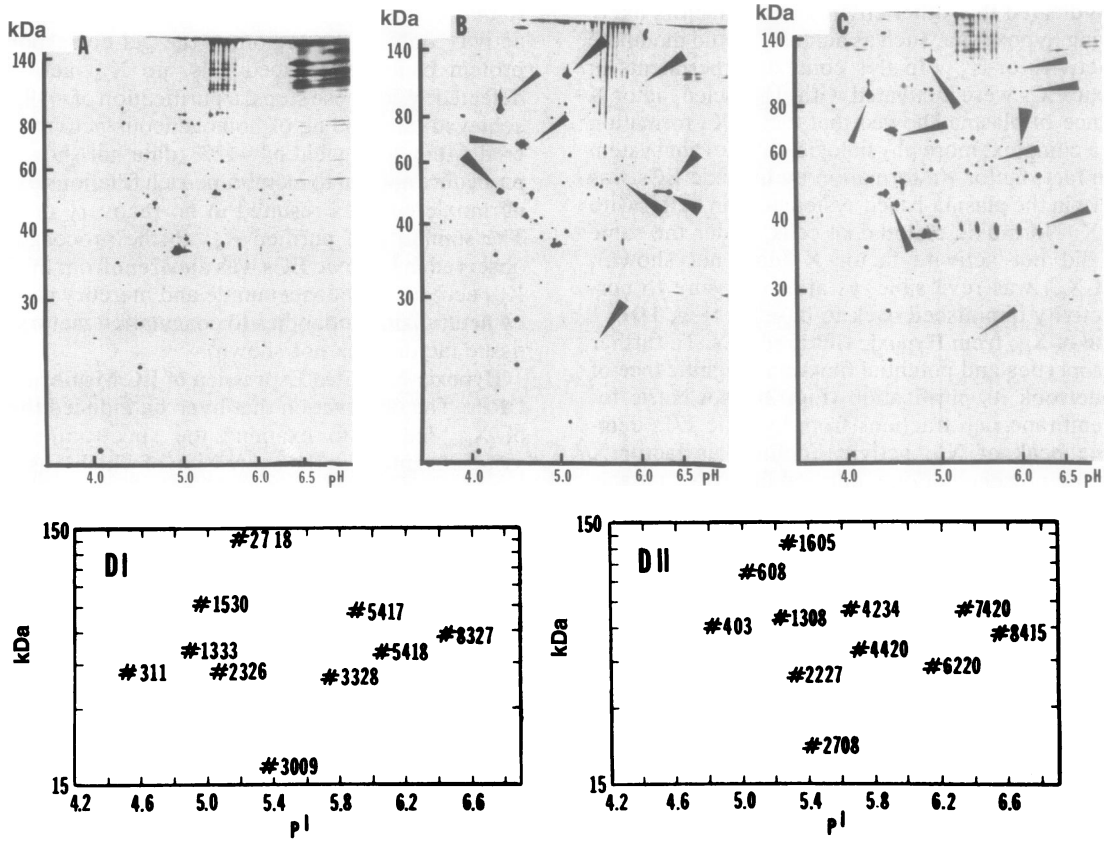

FIG. 3. Autoradiograms and difference maps from two-dimensional gels of membranes derived from hypoxic and normoxic endothelial cultures. ECs were grown to confluence in normoxia and then either maintained in normoxia for 24 hr (*A*), or placed in hypoxia ($pO_2 \approx 14$ mmHg) for 24 hr (*B*) or 48 hr (*C*). [^{35}S]Methionine was added to cultures 8 hr before harvesting samples, EC membranes were prepared, and samples were processed for SDS/PAGE. Arrowheads (*B* and *C*) in hypoxic gels denote spots enhanced (>five times intensity in normoxic controls) or induced in hypoxia. (*D*) Difference map, based on PDQUEST analysis and visual inspection of 20 two-dimensional gel experiments: early ORPs are shown in *DI*, and late ORPs are shown in *DII*.

To know whether hypoxia was the specific stimulus that modulated ORP expression, it was important to know whether ORPs were expressed in response to other recognized cellular perturbations, such as heat shock, glucose deprivation, or TNF exposure (in the latter case, data not shown). Two-dimensional gel analysis of EC membranes after each of the latter three perturbations demonstrated a complex pattern. With the PDQuest system, difference maps of proteins the expression of which was enhanced or induced were constructed (Fig. 4*A–B*); no overlap with hypoxia was seen.

To examine metabolic pathways that could be involved in the production of ORPs, ECs were incubated with either fluoride, 2-deoxyglucose, or azide, and two-dimensional gel analysis was done (Fig. 4 *C–E*). Each inhibitor induced different patterns of membrane-associated proteins, but only azide (Fig. 4*E*) induced spots identical to ORPs (311, 403, 608, and 7420), suggesting that inhibition of the respiratory chain may be involved in the expression of these ORPs.

DISCUSSION

Hypoxemia, which is frequently associated with a range of cardiovascular and pulmonary disorders, is a pathophysiologically relevant example of a common perturbation of the endothelial microenvironment. We report here the purification of an activator of coagulation factor X that is induced by hypoxia, termed tentatively X_{act}. From our current evidence, X_{act} appears distinct from tissue factor and the factor IXa–VIIIa complex. Furthermore, migration of the 100-kDa X_{act} on SDS/PAGE is distinct from that of the 68-kDa tumor procoagulant (16), an activator of factor X present in malignant tissue that bears certain similarities to X_{act}, including inhibition by mercury chloride and iodoacetamide, and ability to be extracted from membranes by low-ionic strength Veronal buffer. Although insufficient X_{act} was available for formal kinetic experiments, its effectiveness for activation of factor X at pH 7.4 appeared low; X_{act} is about two orders of magnitude less efficient than tissue factor-mediated factor X activation on thrombin-perturbed endothelium (17). The low activity of X_{act} suggests that other coagulation factors or plasma components may be more effective substrates for this enzyme. One means of enhancing the reactivity of X_{act} for factor X involved reduction of the pH of the reaction mixture, suggesting that X_{act} may function more effectively in an ischemic milieu where acidosis and, perhaps, other components of the altered environment may further promote its activity. In addition, when factor X activation was studied on hypoxic ECs in the presence of other plasma proteins, ≈3-fold enhancement in factor Xa formation occurred, implying that other components of the plasma may regulate activity of X_{act}.

Work by other investigators indicating that anoxia induced the synthesis of ORPs by other cell types (5, 6) led us to define ORPs in endothelium. From analysis of many two-dimensional gels using the PDQuest system, our studies demonstrate that hypoxia induces the expression of at least 16 membrane-associated proteins in ECs, compared with normoxic controls. Studies of total cell lysates and released products of anoxic fibroblasts (6), with methods similar to ours, did not identify spots/proteins resembling those seen with endothelium. In addition, we note (*i*) the absence of any similarity between the pattern of membrane-associated proteins induced in ECs subjected to heat shock, glucose dep-

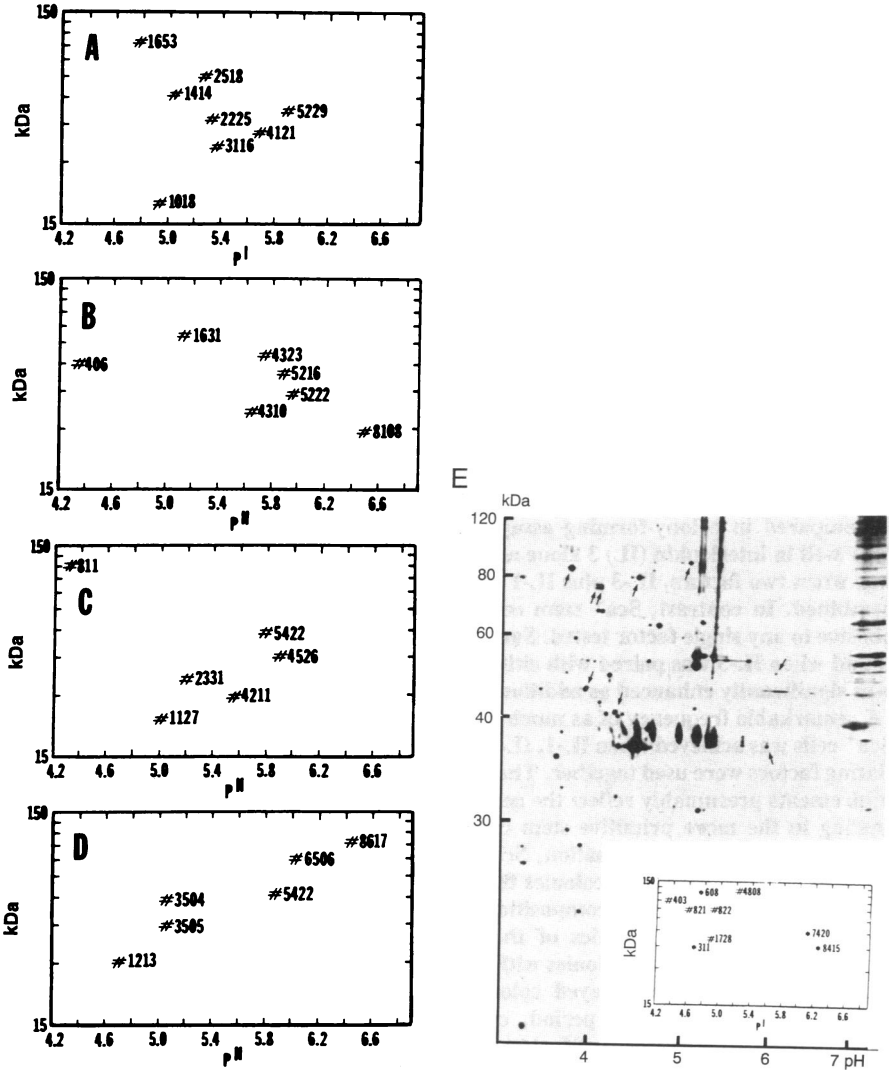

FIG. 4. Comparison of hypoxia-induced endothelial membrane-associated proteins with those induced by heat shock, glucose deprivation, TNF, and metabolic inhibitors. ECs were exposed to heat shock (*A*; 42°C for 3 hr), glucose-free medium (*B*; 16 hr), fluoride (*C*; 1 mM for 16 hr), 2-deoxyglucose (*D*; 25 mM for 16 hr), or azide (*E*; 1 mM for 16 hr) and subjected to two-dimensional gel analysis. Difference maps of spots enhanced in expression (>five times) or induced are shown. Because spots with identical mobility to ORPs were found in *E*, the autoradiogram is displayed (arrows indicate enhanced spots) as well as a difference map (*Inset*).

rivation, or TNF and those observed in hypoxia; and (*ii*) hypoxia of vascular smooth muscle cells did not induce proteins that comigrated with the EC membrane-associated ORPs (data not shown), both suggesting that a component of the EC biosynthetic response to hypoxia appears to involve the production of distinctive proteins.

Our studies indicate that a part of the EC response to hypoxia includes induction of the synthesis of a range of additional or modified proteins that can contribute to the perturbed functional phenotype of hypoxic cultures. X_{act} is an example of a protein induced in the presence of only low oxygen concentrations that could potentially locally activate the coagulation system in ischemic vasculature and mark hypoxemic vessels.

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1. Stelzner, T., O'Brien, R., Sato, K. & Weil, J. (1988) *J. Clin. Invest.* **82,** 1840–1847.
2. Hamer, J., Malone, P. & Silver, I. (1981) *Br. J. Surg.* **68,** 166–170.
3. Lee, S.-L. & Fanburg, B. (1987) *Circ. Res.* **60,** 653–688.
4. Ogawa, S., Gerlach, H., Esposito, C., Pasagian-Macaulay, A., Brett, J. & Stern, D. (1990) *J. Clin. Invest.* **85,** 1090–1098.
5. Subjeck, J. & Thung-Tai, S. (1986) *Am. J. Physiol.* **250,** C1–C17.
6. Anderson, G., Stoler, D. & Scarcello, L. (1989) *J. Biol. Chem.* **264,** 14885–14892.
7. Loike, J., Kozler, V. & Silverstein, S. (1979) *J. Biol. Chem.* **254,** 9558–9564.
8. Madri, J., Partt, B. & Tucker, A. (1988) *J. Cell Biol.* **106,** 1375–1384.
9. Bajaj, P. & Mann, K. (1973) *J. Biol. Chem.* **248,** 7729–7741.
10. Silverberg, S., Nemerson, Y. & Zur, M. (1977) *J. Biol. Chem.* **252,** 8481–8488.
11. Laemmli, U. (1970) *Nature (London)* **227,** 680–685.
12. Henderson, L. & Konigsberg, W. (1979) *Anal. Biochem.* **93,** 153–157.
13. O'Farrell, P. (1975) *J. Biol. Chem.* **250,** 4007–4021.
14. Garrels, J. (1989) *J. Biol. Chem.* **264,** 5283–5298.
15. Mann, K. (1984) *Prog. Hemostasis Thromb.* **2,** 1–23.
16. Falanga, A. & Gordon, S. (1985) *Biochemistry* **24,** 5558–5567.
17. Almus, F., Rao, L., Mohan, V. & Rapaport, S. I. (1989) *Thromb. Haemostasis* **62,** 1067–1073.