The effect of anoxia and reoxygenation on the synthesis and secretion of tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor-1 (PAI-1) was studied in primary cultures of human umbilical vein endothelial cells. Sublethal anoxia, determined by trypan blue dye exclusion and lactate dehydrogenase release, was produced by cell culture under a 95% N₂, 5% CO₂ atmosphere for 2–24 h and was followed by reoxygenation with 95% air, 5% CO₂ for 24 or 48 h. Anoxia did not alter the level of t-PA mRNA or PAI-1 mRNA in the cells or the secretion of t-PA or PAI-1 into the medium. At 24 h, t-PA secreted into conditioned medium was 7.0 ± 1.4 ng/2 × 10⁶ cells (n = 9) and PAI-1 was 300 ± 13 IU/2 × 10⁶ cells (n = 9), whereas the content of t-PA mRNA was 2.2 pg/μg of RNA and PAI-1 mRNA was 180 pg/μg of RNA. During reoxygenation, however, t-PA antigen and PAI-1 activity increased significantly to reach 27 ± 1.0, 49 ± 2.0, and 47 ± 14% of control values, respectively, within 24 h of anoxia. t-PA mRNA also decreased significantly during reoxygenation following anoxia, but the extent could not be accurately quantitated. Addition, during anoxia, of a 200 μg/ml concentration of the superoxide anion radical scavenger superoxide dismutase or of a 5 mM concentration of the iron chelator deferoxamine mesylate prevented the subsequent decrease of t-PA antigen during reoxygenation; addition of these compounds during reoxygenation had no effect. Superoxide dismutase, but not deferoxamine mesylate, when added during anoxia prevented the subsequent decrease in PAI-1 activity. These studies suggest that the marked alteration of endothelial cell fibrinolysis during anoxia followed by reoxygenation is most likely mediated by a mechanism dependent on oxygen radicals. Impaired endothelial cell fibrinolysis may contribute to the pathophysiology of ischemia/reperfusion injury.

Human vascular endothelial cells play an active role in the regulation of fibrinolysis through the synthesis and secretion of both tissue-type plasminogen activator (t-PA)¹ and plasminogen activator inhibitor-1 (PAI-1) (1, 2). Elucidation of mechanisms which modulate the production of these proteins by the endothelium may contribute to the understanding of the pathogenesis of thromboembolic disease (3).

The human vascular endothelium is a target as well as a source of oxidants during ischemia/reperfusion injury (4, 5). Endothelial cells subjected to culture conditions with low oxygen tension produce free radicals upon reoxygenation (6), whereas the hypoxic bovine endothelium has a reduced synthesis of plasminogen activators and an increased synthesis of plasminogen activator inhibitors (7).

In this study, we have investigated the effect of sublethal anoxia followed by reoxygenation on the regulation of human vascular endothelial cell fibrinolysis at both the protein and mRNA levels and have defined the role of oxygen radicals in this phenomenon.

MATERIALS AND METHODS

Reagents—Collagenase (type 1) and catalase were obtained from Worthington; rabbit anti-factor VIII and fluorescein-labeled goat anti-rabbit IgG were from Cappel Laboratories (Cochraneville, PA); medium 199, fetal bovine serum, calf serum, neomycin, nystatin, basic Eagle's medium, vitamins, amino acids, penicillin/streptomycin, t-glutamine, Hanks' balanced salt solution, and trypan blue were from GIBCO; Pentex bovine serum albumin (fraction V, fatty acid-poor) was from Miles Laboratories Inc. (Naperville, IL); protein assay dye reagent was from Bio-Rad; HEPES was from United States Biochemical Corp.; deferoxamine mesylate was from Boehringer Mannheim; deferoxamine mesylate was from Ciba-Geigy; RNA calibration mixture was from Gibco/Bethesda Research Laboratories; and β-nicotinamide-adenine dinucleotide, fluorescein diacetate, 3-amino-1,2,4-triazole, superoxide dismutase, cycloheximide, and all other reagents, unless otherwise specified, were obtained from Sigma.

Cell Culture—Primary cultures of human umbilical vein endothelial (HUVE) cells were established as previously described (8–10) using a brief collagenase digestion (0.1%, w/v in phosphate-buffered saline). Cells were plated into 60-mm culture dishes at a seeding density of 1.5 × 10⁴ cells/dish in 4 ml of medium 199 supplemented with 15% fetal calf serum, 2 mM L-glutamine, 100 μg/ml neomycin, and 20 units/ml nystatin and were grown to confluence at 37 °C in a humidified atmosphere of 95% air, 5% CO₂. Confluent cultures at a density of ~2 × 10⁶ cells/well exhibited the cobblestone morphology typical of endothelial cells.
characteristic of endothelium, and >99% of these cells contained factor VIII-related antigen as determined by indirect immunofluorescence (11) using rabbit anti-factor VIII antisera and fluorescein-labeled goat anti-rabbit IgG.

Experimental Protocols. Anoxic culture conditions (0.5% O2, 5% CO2) were maintained using an Oxysizer (Model 311, Reming Bioinstruments, Redfield, NY) (12, 13) which maintained the oxygen level at a calibrated 0.1% throughout the experiment. The cells were rinsed twice with Hanks' balanced salt solution; refed with serum-free medium 199 containing 0.35% bovine serum albumin and 20 mM HEPES, pH 7.4, and incubated under anoxic conditions for 2-24 h. Following anoxia, the spent medium was collected for analysis and replaced with fresh medium. Cells were then reinduced to normoxic conditions, i.e. 95% air, 5% CO2 (reoxygenation) for 24 or 48 h. The conditioned medium was collected, centrifuged at 100,000 g to remove cellular debris, and stored in a final concentration of phosphate-buffered saline, 0.01% Tween 80 at -20 °C until used for determination of t-PA and PAI-1.

Determination of Oxidant Injury.—The sublethal effects of anoxia and subsequent reoxygenation were determined at designated time points by morphological observations using light microscopy and viability studies consisting of trypan blue dye exclusion (14) and retention of fluorescein diacetate (15). One thousand cells from at least 10 representative fields in the culture vessel were screened. The release of lactate dehydrogenase into the medium was quantitated as described elsewhere (16).

Assay Techniques. The concentration of t-PA was measured by enzyme-linked immunosorbent assay as described (17) and expressed in nanograms/milliliter by comparison with the International Reference Preparation for t-PA, which contains 2 µg/vial. The concentration of urokinase-type plasminogen activator antigen was measured by two-site enzyme-linked immunosorbent assay as described (18). PAI-1 activity was determined by the method of Verheijen et al. (19) and expressed in international units of t-PA neutralized by comparison with the International Reference Preparation for t-PA, which contains 1000 IU/vial. PAI-1, which is spontaneously converted to a latent form (20) after secretion into cell culture medium, was reacted with neutralized SDS. Fibrinolytic activity was measured on plasminogen-containing bovine fibrin plates by the method of Astrup and Mullertz (21). Total protein concentration present in the cell-free medium was determined by the Bio-Rad protein assay kit (22).

Additional Protocols. Superoxide dismutase (EC 1.15.1.1) (bovine erythrocyte-derived; 3000 units/mg of protein; CuZn form; 200 µg/ml) was added either at the onset of anoxia or prior to reoxygenation.

The specificity of the effect was evaluated with the use of 200 µg/ml inactivated superoxide dismutase. Superoxide dismutase was inactivated by incubation with H2O2 at pH 10 (23). HUVE cells were treated with 5 mM deferoxamine mesylate in medium 199 24 h prior to anoxia, during anoxia, or at the onset of reoxygenation.

Lactate levels were measured in protein-free cell conditioned medium and in cell lysates of control and anoxic HUVE cells (12- and 24-h exposure) using a modification of the assay described by Gurman et al. (20). The sample (100 µl) was incubated in an assay mixture consisting of 0.5 M glycine, 0.4 M hydrabuse buffer, pH 9.0, 40 mM β-NAD, and 5 mg/ml lactate dehydrogenase (550 units/mg of protein) in a final volume of 1.5 ml. Values were compared to a standard curve ranging from 0.025 to 0.3 mM lactate. Absorbance at 340 nm was measured before and after 30 min at 37 °C. Cell lysates were prepared by incubation of cell monolayer with 0.25% Triton X-100 in phosphate-buffered saline for 15 min at 37 °C, followed by centrifugation at 10,000 x g.

Quantitation of t-PA and PAI-1 mRNA Levels by Slot-Blot and Northern Blot Analyses.—Total cellular RNA was extracted from cells by lysis with 1 ml of 5 mM guanidinium isothiocyanate/well, 25 mM sodium citrate, pH 7.0, containing 0.5% Sarkosyl and 8% β-mercaptoethanol as described by Chomczynski and Sacchi (27), followed by cold phenol extraction (performed only once). The final RNA pellet was resuspended in 100 µl of H2O, and the concentration was determined by absorbance at 260 nm.

RNA was denatured with 6 M guanidyl cleavage at 50 °C; 2.0, 1.0, or 0.5 µg was applied to a nylon membrane (Zetaprobe, Bio-Rad) according to the manufacturer's instructions using a slot blot apparatus (Schleicher & Schuell). A standard curve of known concentrations of t-PA or PAI-1 mRNA was applied to each membrane. The t-PA and PAI-1 mRNAs used for the construction of these calibration curves were prepared using an in vitro SP6 polynucleotide system (Riboprobe, Promega Biotech) and quantitated by absorbance at 250 nm (28). Therefore, a 2000-base pair-long PAI-1 cDNA, containing most of the coding sequence and 700 base pairs of the 3' untranslated sequence (29), was cloned into nSp65. Alternatively, the BglII cDNA fragment of t-PA (nucleotides 230-2265), according to the published t-PA cDNA sequence (30), was cloned into pGem3 containing nucleotides 616-1015 of β-actin according to the published cDNA sequences (29-31). The probes were prepared freshly using a Promega Biotech transcription kit and routinely had a specific activity of 0.5-2.0 x 107 cpm/µg of RNA. The probes were heat-denatured and added to the hybridization mixture at a concentration of 106 cpm/ml. Membrane washing conditions at 65 °C were as described elsewhere (32).

**Table I**

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>Culture conditions</th>
<th>Total protein</th>
<th>Antigen PAI-1 activity</th>
<th>Before reactivation</th>
<th>After reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 h</td>
<td>Control</td>
<td>0.78 ± 0.01</td>
<td>&lt;1</td>
<td>70 ± 8</td>
<td>360 ± 41</td>
</tr>
<tr>
<td>6 h</td>
<td>Anoxic</td>
<td>0.83 ± 0.14</td>
<td>&lt;1</td>
<td>87 ± 43</td>
<td>440 ± 70</td>
</tr>
<tr>
<td>12 h</td>
<td>Anoxic</td>
<td>0.82 ± 0.05</td>
<td>0.9 ± 0.5</td>
<td>110 ± 26</td>
<td>650 ± 170</td>
</tr>
<tr>
<td>24 h</td>
<td>Anoxic</td>
<td>0.91 ± 0.25</td>
<td>1.3 ± 0.2</td>
<td>100 ± 25</td>
<td>640 ± 90</td>
</tr>
<tr>
<td></td>
<td>Anoxic</td>
<td>0.88 ± 0.06</td>
<td>4.4 ± 0.6</td>
<td>230 ± 17</td>
<td>1100 ± 160</td>
</tr>
<tr>
<td></td>
<td>Anoxic</td>
<td>0.85 ± 0.06</td>
<td>3.9 ± 0.8</td>
<td>220 ± 10</td>
<td>1200 ± 270</td>
</tr>
<tr>
<td></td>
<td>Anoxic</td>
<td>0.96 ± 0.18</td>
<td>6.3 ± 0.8</td>
<td>300 ± 63</td>
<td>3100 ± 310</td>
</tr>
</tbody>
</table>

**Table I** t-PA antigen and PAI-1 activity secreted by control and anoxic HUVE cells. The data represent the mean ± S.D. of three experiments performed in triplicate and are expressed per 2 x 106 cells.
Table II

<table>
<thead>
<tr>
<th>t-PA antigen</th>
<th>Before reactivation</th>
<th>After reactivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI-1 activity</td>
<td>ng/ml</td>
<td>IU/ml</td>
</tr>
<tr>
<td>24 h reoxygenation</td>
<td>Anoxia</td>
<td>7.0 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>5.4 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>8 h</td>
<td>3.9 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>1.9 ± 0.1</td>
</tr>
<tr>
<td>48 h reoxygenation</td>
<td>Anoxia</td>
<td>13.0 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>0 h</td>
<td>9.2 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>8 h</td>
<td>8.0 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>12 h</td>
<td>6.5 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>24 h</td>
<td>4.5 ± 0.2</td>
</tr>
</tbody>
</table>

RESULTS

Effect of Anoxia—The effect of anoxia on the viability of primary cultures of HUVE cells was determined using trypan blue dye exclusion and fluorescein diacetate inclusion. Both control and anoxic cell cultures remained essentially 100% viable following 2, 8, and 24 h of exposure. Lactate dehydrogenase activity in medium conditioned by control and anoxic/reoxygenated cells was 3.4 ± 3 and 3.0 ± 3 milliunits (mean ± S.D.) per 2 x 10^6 cells/ml of medium, respectively. Lactate dehydrogenase activity (release) in both control and anoxic/reoxygenated cell conditioned medium represented <2% of the total lactate dehydrogenase activity present within the endothelial cells. The protein content of lysed cultures from control and anoxic cells was not significantly changed (<1 mg/ml). These data imply that 24 h of exposure to 96% N_2, 4% CO_2 is sublethal for cultured HUVE cells.

Lactate production after 24 h of incubation was measured both in protein-free conditioned medium and in cell lysates. Control cultures contained 11 ± 0.1 mM (mean ± S.D., n = three to six samples for three experiments) and 0.98 ± 0.005 mM, respectively; and anoxic cultures contained 15 ± 0.5 and 0.19 ± 0.6 mM, respectively. Thus, anoxia resulted in significant increases (p < 0.05) in the lactate levels in conditioned medium as well as in cell extracts.

t-PA antigen secretion and PAI-1 activity (active and latent) were determined in cell-free conditioned medium from HUVE cells grown under normal and anoxic incubation conditions. Medium was harvested and analyzed following 2, 6, 12, and 24 h of culture. These results are summarized in Table I. No significant difference in either t-PA antigen secretion or PAI-1 activity was detected between control and anoxic cultures at any of the time points tested. In both cases, t-PA antigen and PAI-1 activity increased concomitantly with time in culture. PAI-1 mRNA levels during anoxia did not significantly differ from control levels (data not shown), whereas t-PA mRNA levels were below the sensitivity level of our assay. Protein determinations after 2, 6, 12, and 24 h of culture showed no change in cell protein content following exposure to normoxia and anoxia. No urokinase-type plasminogen activator antigen was detected in either control or anoxic cultures at any time point.

Effects of Reoxygenation—Reoxygenation during 24 h following anoxia lasting 2–24 h had no effects upon cell viability, lactate dehydrogenase release, or total protein content. Again, cell viability was essentially 100%; lactate dehydrogenase release was <1 IU/ml, and total cell protein content at 2, 6, 12, and 24 h remained unchanged between normoxic and reoxygenated cultures (data not shown).

Re-exposure of HUVE cells to normal culture conditions,

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**FIG. 1. Immunoprecipitation and Western blotting of t-PA antigen from conditioned medium from cultured endothelial cells.** Lane A, purified t-PA; lane B, control HUVE cells; lane C, HUVE cells exposed to anoxia; lane D, HUVE cells exposed to 24 h of anoxia followed by 24 h of reperfusion. Molecular weight standards are indicated by the numbers at the left. The extra bands with apparent M, values of 200, 75, and 60 which appear on the blot are most likely due to detection of immunoglobulin dissociated from Sepharose during immunoprecipitation since they were also observed in unconditioned medium. t-PA antigen was recovered exclusively with M, = 110,000 consistent with t-PA PAI-1 complex formation.

**FIG. 2. Effect of anoxia/reoxygenation on t-PA and PAI-1 mRNA levels.** A, Northern blot analysis of total cellular RNA isolated from HUVE cells exposed to either control/control (C/C) or anoxia/reoxygenation (A/R) (24 h of anoxia followed by reoxygenation for 24 h). A, samples probed for PAI-1 and β-actin mRNAs; B, samples probed for t-PA and β-actin mRNAs. The molecular size, expressed in kilobases (kb), was determined using the RNA calibration mixture. B, total cellular RNA was isolated from control/control (C/C) and anoxia/reoxygenated (A/R) cells and assayed for PAI-1 and β-actin mRNAs by slot blot analysis. The slots are from a single experiment representative of three individual experiments. For details, see "Materials and Methods."
i.e. 95% air, 5% CO₂ for 24 or 48 h, after a period of anoxia between 2-24 h had significant (p < 0.05) effects upon both t-PA antigen secretion and PAI-1 activity (Table II). t-PA antigen secretion decreased markedly in HUVE cells during 24 h of reoxygenation proportionally to the length of the previous period of anoxia. The level in control cells at each time point did not significantly differ from 7.0 ± 1.4 ng of t-PA antigen/ml of medium, whereas cells pre-exposed to 2 h of anoxia secreted 5.4 ± 1.7 ng/ml within 24 h of reoxygenation, and those subjected to 8-24 h of anoxia secreted 3.9 ± 0.5 ng/ml following reoxygenation, respectively.

PAI-1 activity, measured both before and after reactivation, also decreased following reoxygenation for 24 h and was likewise dependent upon the previous period of anoxia. Anoxia followed by 48 h of reoxygenation had a significant effect upon the secretion of t-PA antigen, which remained markedly reduced. However, PAI-1 activity (both active and latent) had meanwhile recovered to levels seen in control cells.

SDS gel electrophoresis and immunoblotting with anti-t-PA IgG (Fig. 1) of 24-h conditioned medium (lane B) revealed no antigen co-migrating with free t-PA (lane A), but revealed a band migrating with an apparent Mr of 110,000, compatible with the t PA-1 complex. This band did not change during 24 h of anoxia (lane C), but was markedly reduced in conditioned medium obtained after 24 h of reoxygenation following 24 h of anoxia (lane D). The immunoblot of conditioned medium from control cells exposed to normoxia for 24 h followed by an additional 24 h of normoxia did not differ significantly from the immunoblot of medium exposed to normoxia for 24 h seen in lane B (data not shown).

Fig. 2A shows results of Northern blot analysis of total cellular RNA obtained from HUVE cells exposed to either normoxic or anoxic conditions for 24 h followed by 24 h of reoxygenation. A marked decrease in t-PA mRNA levels (2.8 kilobases) and in both PAI-1 mRNA species (3.4 and 2.4 kilobases) was observed. Hybridization with a β-actin RNA probe showed that equivalent amounts of RNA had been applied to the gel and that β-actin mRNA (1.8 kilobases) levels were not changed by anoxic/reoxygenation treatment. Quantitation of PAI-1 mRNA levels, by slot blot analysis after standardization against β-actin mRNA levels to correct for any differences in application to the nitrocellulose membrane, showed that PAI-1 mRNA levels fell from 190 ± 13 to 100 ± 19 pg/μg of RNA after reoxygenation (Fig. 2B). t-PA mRNA levels from anoxic/reoxygenated cells were very low, not allowing accurate quantitation.

Effect of Oxygen Scavengers—Superoxide dismutase, a scavenger of the superoxide anion radical, was added to a final concentration of 200 μg/ml to the cell incubation medium prior to anoxia and, in separate experiments, at the onset of reoxygenation (Table III). Superoxide dismutase effectively restored t-PA antigen secretion to that of controls after 24 h reoxygenation when present during the anoxic period. When superoxide dismutase was added at the onset of reoxygenation, no change in t-PA antigen secretion occurred. In addition, superoxide dismutase which had been catalytically inactivated had no ameliorative effect upon the synthesis and secretion of t-PA. This was irrespective of its addition either at the onset of anoxia or immediately before reoxygenation, thus demonstrating the specificity of the protection afforded by superoxide dismutase in our test system.

It has become evident that the ionic species of iron (Fe²⁺, Fe³⁺) function in both the initiation and catalysis of a variety of free radical reactions that contribute to oxygen-dependent cellular injury. Iron ions are free radicals (32) capable of participating in single electron transfer reactions with molecular oxygen. The generation of O₂⁻ by any source in the presence of iron ions can lead to the formation of hydroxyl radicals (OH⁻) by modified Fenton chemistry (33). The mechanism(s) by which iron-mediated damage occurs in vivo is not fully understood. Evidence has accumulated indicating that oxidative cell damage is enhanced by submicromolar to micromolar levels of non-ionic iron, presumably in equilibrium with iron sequestered in ferritin pools (34).

The effects of deferoxamine mesylate, an iron chelator known to prevent the formation of hydroxyl radicals (35), when added before anoxia, at the onset of anoxia, or immediately prior to reoxygenation are also summarized in Table III. Deferoxamine mesylate was effective in preventing the t-PA antigen decrease when added before or during anoxia, but not at the onset of reoxygenation. Deferoxamine mesylate did not, however, block the PAI-1 activity decrease, irrespective

### Table III

Effect of oxygen scavengers on the secretion of t-PA and PAI-1 during reoxygenation

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>t-PA antigen</th>
<th>PAI-1 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/ml</td>
<td>IU/ml</td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td></td>
<td>reactivation</td>
<td>reactivation</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.6 ± 0.5</td>
<td>340 ± 70</td>
</tr>
<tr>
<td>24 h anoxia/24 h reoxygenation</td>
<td>0.75 ± 0.06</td>
<td>150 ± 17</td>
</tr>
<tr>
<td>24 h anoxia with SOD/24 h reoxygenation</td>
<td>2.6 ± 0.6</td>
<td>350 ± 90</td>
</tr>
<tr>
<td>with inactivated SOD/24 h reoxygenation</td>
<td>0.78 ± 0.05</td>
<td>160 ± 20</td>
</tr>
<tr>
<td>24 h anoxia/24 h reoxygenation with SOD</td>
<td>0.70 ± 0.01</td>
<td>290 ± 119</td>
</tr>
<tr>
<td>24 h anoxia/24 h reoxygenation with DES</td>
<td>0.76 ± 0.03</td>
<td>155 ± 21</td>
</tr>
</tbody>
</table>

* SOD, superoxide dismutase; DES, deferoxamine mesylate; ND, not detectable.
of its addition immediately prior to either anoxia or reoxygenation.

DISCUSSION

The vascular endothelial cells assume a key role in the regulation of fibrinolysis through the synthesis and secretion of both t-PA and PAI-1 (1, 2). In this study, we report that culture under anoxia and reoxygenation conditions, designed to simulate sublethal ischemia/reperfusion injury, had significant effects upon human endothelial cell fibrinolysis at both the antigen and mRNA levels. When human endothelial cells were cultured in an anoxic environment for as long as 24 h, no effect on the synthesis and secretion of t-PA or PAI-1 was observed. However, during subsequent reoxygenation, a significant decrease in both t-PA and PAI-1 secretion was observed which was proportional to the length of the previous period of anoxia. t-PA and PAI-1 mRNA levels also decreased during reoxygenation, indicating that the reduction in t-PA and PAI-1 secretion reflects a decrease in transcription. The superoxide anion radical scavenger superoxide dismutase and the iron chelator deferoxamine mesylate prevented the decrease in t-PA secretion, but only when they were present during the anoxic phase. In experiments in which cells were treated for 24 h with either superoxide dismutase (data not shown) or deferoxamine mesylate (Table III), rinsed repeatedly, and then cultured under anoxic/reoxygenation conditions, only pretreatment with deferoxamine mesylate was effective in preventing the decrease in t-PA antigen secretion. These data presumably reflect uptake of deferoxamine mesylate by the endothelial cell (35), which in turn renders the cell resistant to injury induced by anoxia/reoxygenation. The ineffectiveness of pretreating endothelial cells with superoxide dismutase suggests that the efficacy of superoxide dismutase in this system is not mediated by its entry into the cell.

The results of our study, which showed no measurable effect of anoxia on endothelial cell fibrinolysis, differ from those of previously reported studies. Two reports have documented changes in endothelial cell fibrinolysis which occurred as a direct consequence of anoxic culture conditions. Wojta et al. (7), using bovine microvascular and pulmonary artery endothelial cells, have shown that anoxic culture conditions were responsible for a decrease in t-PA activity and an increase in PAI-1 activity. Moreover, subsequent reoxygenation of these cultures was not effective in reversing the depression in t-PA activity observed in these cells. These studies are in contrast to those of Bach et al. (36), who showed that anoxic culture conditions caused a 6-fold increase in t-PA antigen levels in bovine endothelium cells and a 28% increase in HUVE cells. In addition, they found PAI-1 activity to be decreased in human cells. These differences may reflect variations in culture conditions as well as in the capability to monitor anoxia.

Alterations in endothelial cell fibrinolysis occur only during reoxygenation, but they are clearly dependent upon the length of the previous period of anoxia. Moreover, this process is probably mediated by oxygen radicals because the addition of radical scavengers during the period of anoxia prevents the subsequent changes. Our findings are in agreement with in vivo reports which suggested an active participation of oxygen free radicals in ischemic injury to both the central nervous system (37) and intestine (38). Model systems using isolated hearts suggested that the reintroduction of oxygen to tissue subjected to previous hypoxia is detrimental (39). It has been demonstrated by several investigators (40, 41) that anoxia produces a loss of thiol groups and glutathione, which causes tissue membrane peroxidation and leads to a reduction in myocardial superoxide dismutase and glutathione peroxidase activities. These changes were substantially increased by reperfusion, indicating that reoxygenation can cause free radical damage and alterations in protective antioxidant enzymes. Several studies have also shown that oxygen radical scavengers, when added to these test systems, reduce oxidant-mediated ischemia/reperfusion injury (42-44).

This study further supports the active involvement of oxygen free radicals in alterations in cell and organ dysfunction induced by ischemia/reperfusion. The understanding of endothelial cell fibrinolytic alterations by anoxia and reoxygenation may be clinically relevant. Indeed, differential recovery of PAI-1 and t-PA production after reperfusion may play a role in the reocclusion phenomenon which may occur following successful lysis of coronary artery thrombosis in ischemic heart disease. An understanding of mechanisms governing interventions with oxygen radical scavengers during the period of anoxia may be a key in restoring endothelial cell fibrinolytic potential and therefore preventing subsequent blood vessel reocclusion.

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Oxygen radicals generated during anoxia followed by reoxygenation reduce the synthesis of tissue-type plasminogen activator and plasminogen activator inhibitor-1 in human endothelial cell culture.

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