Modulation of the Fibrinolytic Response of Cultured Human Vascular Endothelium by Extracellularly Generated Oxygen Radicals*

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The fibrinolytic system is regulated by two plasminogen activators (PAs), tissue-type plasminogen activator (t-PA) antigen, plasminogen activator inhibitor-1 (PAI-1) antigen, and PAI-1 activity were determined in 1.25 ml of conditioned medium and t-PA and PAI-1 mRNA in the cell extracts of 2 × 10⁶ HUVE cells. Control cells secreted 3.9 ± 1.3 ng/ml (mean ± S.D., n = 12) within 24 h. Treatment with xanthine/xanthine oxidase for 15 min induced a 2.8 ± 0.4-fold increase (n = 12, p < 0.05) of t-PA antigen secretion after 24 h. The t-PA antigen was recovered predominantly in complex with PAI-1. The oxidant injury caused a 3.0 ± 0.8-fold increase (n = 9, p < 0.06) in t-PA mRNA within 2 h. Total protein synthesis was unaltered by xanthine/xanthine oxidase. The oxidant scavengers superoxide dismutase and catalase, in combination, abolished the effect of xanthine/xanthine oxidase on the expression of components of the fibrinolytic system in primary cultures of human umbilical vein endothelial (HUVE) cells. The results show that the synthesis and secretion of t-PA antigen and mRNA but not of PAI-1 antigen and mRNA are increased following exposure to oxidants.

**MATERIALS AND METHODS**

**Reagents**—Collagenase (type-1), and catalase were obtained from Worthington; rabbit anti-factor VIII and fluorescein-labeled goat anti-rabbit IgG from Cappel Laboratories, Cochranville, PA; Medium-M199, fetal bovine serum, calf serum, neomycin, nystatin, basal medium Eagle vitamin solution and amino acids, trypsin, Hanks' balanced salt solution, and trypan blue vital dye from Gibco; Chromium from Du Pont-New England Nuclear; xanthine oxidase and histamine from Calbiochem, La Jolla, CA; aprotinin (Trasyol) from FBA Pharmaceuticals, Germany; Pentex bovine serum albumin fraction V, fatty acid-poor, from Miles Laboratories, Inc., Kankakee, IL; Bio-Rad protein assay dye reagent from Bio-Rad; HEPES from United States Biochemical Corp., Cleveland, OH; lactate dehydrogenase from Boehringer Mannheim, Germany; RNA calibration mixture from Gibco/Bethesda Research Laboratories; and fluorescein diacetate, 3-amino-1,2,4-triazole, endotoxin (LPS) from Escherichia coli O55:B5, superoxide dismutase, cycloheximide, xanthine, and all other reagents, unless otherwise specified, were obtained from Sigma.

**Solutions**—Modified medium M-199, pH 7.2–7.4, consisted of 9.87 g/liter medium M-199 powder, 10 ml/liter basal medium Eagle vitamin solution (100 x), 1 g/liter glucose, 0.1 g/liter neomycin (1120 units), 2.2 g/liter NaHCO₃, 0.3 g/liter L-glutamine, 15% fetal bovine serum. Lysing buffer comprised 1 mM EDTA, 5 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 10 units/ml aprotinin, and 1% Triton X-100 at pH 7.4. Tyrode buffer was 137 mM NaCl, 2.7 KCl mm, 11.9 mM NaHCO₃, 0.36 mM NaN₃, 2.9 mM CaCl₂, 1.9 mM MgCl₂, 5.5 mM glucose, 0.55% bovine serum albumin buffer, pH 7.3–7.4; HEPES-Tyrode buffer consisted of Tyrode buffer containing 5 mM HEPES, pH 7.4. PBS-Tween 80 contained 8 g/liter NaCl, 1.6 g/
liter NaHPO4/7H2O, 0.2 g/liter KCl, 0.2 g/liter KH2PO4, pH 7.4, and 0.1% Tween 80.

Cell Culture—Primary cultures of HUVE cells were established as previously described (9, 10) using a brief collagenase digestion (0.1% (w/v) in PBS). Cells were plated into 60-mm culture dishes at a density of 10,000 cells/dish. Leukemia cell-free conditioned medium containing nucleotides 278-885 of t-PA or nucleotides 1045-1481 of PAI-1, which is converted to an inactive form during routine cell culture conditions, was reactivated by the method of Levin and Santell (24) in order to quantitate latent PAI activity. Briefly, samples were made 0.2% in SDS, incubated at 37 °C for 10 min, and then made 2% in Triton X-100, by dialysis prior to assay performance.

Molecular characterization of secretion products was undertaken in the following way. Conditioned medium was harvested and concentrated to 10 to 1 ml by ultrafiltration on a Centricon-30 membrane (Amicon Corp., Lexington, MA). The concentrate was then subjected to gelatinosephorose chromatography with a 5 ml column of molecular weight cut-off Sepharose 4B. For immunoadsorption, 20 μl of a 50% substituted Sepharose slurry was added to 1 ml of media concentrate. Immunoadsorbed t-PA antigen was then eluted by incubation in SDS sample buffer prior to 10% SDS-polyacrylamide gel electrophoresis (25). Fractionated proteins were electrophoretically transferred to nitrocellulose and immunoblotted to the same affinity purified rabbit anti-t-PA IgG (20 μg/ml) by the method of Towbin et al. (26).

Preparation and Isolation of HUVE Cell Extracellular Matrix (ECM)—HUVE ECM were prepared from confluent primary monolayers according to the method of Knudsen et al. (29). The culture medium was first removed and centrifuged at 12,000 × g for 2 min to remove cell fragments and precipitated t-PA. The remaining supernatant was concentrated to 1 ml, made 0.2% in SDS, incubated at 37 °C for 10 min, and then frozen at −20 °C until RNA isolation. The monolayer was then washed three times with PBS. Cells were lysed by treatment with 0.5% Triton X-100 in PBS for 10 min at room temperature, and cell lysates were collected and stored at −80 °C. The cells were washed with PBS and treated with 25 mM NH2OH (approximately 10 min) until light microscopic examination showed the removal of residual cell debris from the surface of the well. The material remaining on the surface after this treatment is defined as ECM. Wells were then washed with PBS and ECM was extracted by scraping into 0.2% SDS in PBS. ECM samples were then made 2% in Triton X-100 and stored at −80 °C.

Quantitation of t-PA and PAI-1 mRNA Levels by Slot Blot and Northern Blot Analysis—Total cellular RNA was extracted from cells by lysis with 1 ml/well of 5 mM guanidinium isothiocyanate, 25 mM sodium citrate, pH 7.0, containing 0.5% Sarkosyl and 8% 8-mercaptoethanol, as described by Chomczynski and Sacchi (30), followed by cold phenol extraction (performed only once). The final RNA pellet was resuspended in 50 μl of H2O and the concentration determined by absorbance at 260 nm.

RNA was denatured with 6 M gloyxal for 1 h at 50 °C and 2,0.0, or 0.5 μg was applied to a nylon membrane (Zetaprobe, Bio-Rad according to the instructions of the manufacturer) using a Branson 250 sonic dismembrane 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 mRNA used for the construction of these calibration curves were prepared using an in vitro SP6 polymerase system (Promega, Leiden, the Netherlands) and quantitated by absorbance at 260 nm (31). A 2000 base pair long PAI-1 cDNA, containing most of the coding sequence, and 700 base pairs of the 3' untranslated sequence (32) was cloned into pSP65. Alternatively, the BglII cDNA fragment of t-PA (nucleotides 230-2205) according to the published t-PA cDNA sequence (33) was cloned into pSP65. Human spleen RNA was used as a control to determine nonspecific hybridization. After application, the membrane was baked at 80 °C for 2 h, vacuum from 37 °C and prehybridized for at least 4 h at 65 °C in the following hybridization mixture: 50% formamide, 5 × SSC (saline-sodium citrate buffer), 10 × Denhardt's solution, 0.5 mg/ml dextran sulfate, 1 mM EDTA, 1% SDS containing heat-denaturing transfer RNA (500 μg/ml) and sonicated, heat-denatured salmon sperm DNA (200 μg/ml).

Hybridization was performed overnight at 65 °C in the same solution containing specific 32P-RNA probes for t-PA mRNA, PAI-1 mRNA, and a noncoding RNA. RNA probes were hybridized to either SP6 polymerase transcription of linearized pSP65 plasmids containing nucleotides 275-885 of t-PA or nucleotides 1045-1481 of PAI-1 or by T7 polymerase transcription of a linearized pGemZ plasmid containing nucleotides 616-1015 of β-actin according to the manufacturer's instructions. RNA probes were quantified by either gel or slot blot analysis using a Promega Transcription Kit (Promega, Leiden, the Netherlands) and had a specific activity of 0.5-2.0 × 1010 cpm/μg RNA. The probes were heat denatured and added to the hybridization
solution at a concentration of 10⁶ cpm/ml. Membrane washing conditions at 65 °C were as described by the manufacturer of Zetaprobe with a final wash of 0.05 × SSC at 65 °C. Autoradiography was carried out using Hyperfilm (Amersham, Brussels, Belgium) at −72 °C. Quantitation was achieved by measuring the radioactivity of each slot by liquid scintillation counting.

For Northern blot analysis, glyoxal-treated RNA (5 or 10 μg) was electrophoresed in a 1% agarose gel followed by capillary transfer to a Zetaprobe membrane. Hybridizations were performed as described for slot blots.

Statistics—Statistical significance of experimental results was determined using the Student's t test for nonpaired comparisons.

RESULTS

Effect of Xanthine/Xanthine Oxidase on the Synthesis and Secretion of Fibrinolytic Components

\[ t-PA -\text{A 15-min exposure of HUVE cells to 50 μM xanthine and 100 milliunits of xanthine oxidase had no significant effect upon HUVE cell viability/cytotoxicity when assayed immediately following oxidant exposure or following a subsequent 24-h period of incubation, as judged from the morphology, trypan blue dye exclusion, fluorescein diacetate inclusion, and chromium or lactate dehydrogenase release. Xanthine/xanthine oxidase treatment resulted in a significant increase in t-PA antigen within 16–32 h, with a 2.8 ± 0.4-fold increase at 24 h (Table I). Histamine, a known agonist of t-PA antigen (35) secretion resulted in a 4–7-fold stimulation (Table II).}]

Addition of xanthine or xanthine oxidase alone did not alter t-PA antigen secretion, higher concentrations or prolonged incubation with the mixture was cytotoxic, and lower concentrations did not produce maximal t-PA secretion. No t-PA activity was detected in the conditioned medium from either control or oxidant-treated cultures on fibrin plates. u-PA antigen was not detected (<0.08 ng/ml) in either the conditioned medium or in cell lysates from either control cells or cells treated with the mixture of xanthine/xanthine oxidase. Total protein concentration present in 24-h cell-conditioned medium was not significantly different between control (0.8 ± 0.3 mg/ml), xanthine/xanthine oxidase– (0.8 ± 0.3 mg/ml), or histamine– (1.0 ± 0.5 mg/ml) treated cells.

Incubation of HUVE cells with 1.0 μg/ml of cycloheximide, a protein synthesis inhibitor, following 15 min of incubation with buffer, histamine, or xanthine/xanthine oxidase, resulted in a marked reduction of t-PA antigen secretion within 24 h (Table II), but did not affect cell viability as assessed by trypan blue dye exclusion.

Western blotting with anti-t-PA antiserum of 24-h conditioned medium (Fig. 1) revealed a main band with a molecular weight of 55,000, corresponding to the increase observed in t-PA antigen synthesis. 

**Table I**

| Incubation time | Culture conditions | t-PA antigen (ng/ml) | PAI-1 antigen (IU/ml) |
|-----------------|-------------------|----------------------|-----------------------|
| h               |                   | Before reactivation  | After reactivation    |
|                 |                   |                      |                       |
| 2               | Control ND        | 300 ± 100            | 25 ± 3                | 83 ± 36                |
|                 | X + XO            | 260 ± 120            | 18 ± 10               | 140 ± 25               |
| 8               | Control ND        | 1,800 ± 700          | 65 ± 32               | 780 ± 180              |
|                 | X + XO            | 2,200 ± 920          | 79 ± 35               | 690 ± 204              |
| 16              | Control 3.3 ± 0    | 9,200 ± 4,400        | 130 ± 45              | 1,210 ± 465            |
|                 | X + XO 8 ± 1      | 12,200 ± 4,000       | 190 ± 28              | 910 ± 140              |
| 24              | Control 4 ± 2      | 7,300 ± 1,100        | 79 ± 9                | 910 ± 70               |
|                 | X + XO 12 ± 2     | 7,100 ± 1,410        | 81 ± 10               | 780 ± 60               |
| 32              | Control 7.7 ± 0    | 4,400 ± 1,570        | 40 ± 26               | 950 ± 300              |
|                 | X + XO 14 ± 0     | 4,600 ± 1,160        | 38 ± 20               | 900 ± 300              |

**Table II**

| Without cycloheximide | With cycloheximide (1.0 μg/ml) |
|-----------------------|--------------------------------|
| ng/ml                 |                                |
| Control               | 3.2 ± 1.0                      |
| Histamine             | 14.0 ± 6.0                     |
| X + XO                | 8.0 ± 1.0                      |

**FIG. 1.** Immunoprecipitation and Western blotting of t-PA antigen from 24-h conditioned medium from cultured HUVE cells. Lane A, control HUVE; lane B, histamine-treated HUVE; lane C, xanthine/xanthine oxidase-treated HUVE. The positions of the molecular weight standards are indicated at the left. The extra band with a molecular weight of 59,000 appears on the blot is most likely due to detection of immunoglobulin dissociated from Sepharose during immunoprecipitation. t-PA antigen was recovered exclusively with M₅ = 150,000 consistent with t-PA–PAI-1 complex formation.
III). When superoxide dismutase (200 μg/ml) and catalase (50 μg/ml) were added individually to the test system, neither was as effective as the combination of superoxide dismutase/catalase in preventing the xanthine/xanthine oxidase induced increase in t-PA antigen secretion by HUVE cells. Chemically or enzymatically inactivated superoxide dismutase and catalase at identical concentrations had no effect on the oxidant-induced stimulation of t-PA antigen secretion (Table III).

Effects of Xanthine/Xanthine Oxidase on mRNA Levels of Fibrinolytic Components

Total cellular RNA was obtained from HUVE cells which had been exposed to xanthine/xanthine oxidase with or without the simultaneous addition of 200 μg/ml superoxide dismutase and 50 μg/ml catalase. Slot blot analysis revealed an increase in t-PA mRNA which reached a peak at 2-h post treatment (3 ± 0.8-fold increase) declining to 1.8 ± 0.1-fold at 8 h and 2.4 ± 0.1-fold at 24 h (Table IV). superoxide dismutase/catalase markedly reduced the effect of xanthine/xanthine oxidase on t-PA mRNA at 2, 8, and 24 h (Table IV).

Fig. 2 shows results of Northern blot analysis of total RNA obtained from HUVE cells harvested 2 h after treatment with xanthine/xanthine oxidase. t-PA mRNA (2.8 kilobases) is barely detectable in control cells (lane A), it is markedly increased in treated HUVE cells (lane B), and this increase

![Image of Northern blot analysis](image)

**Fig. 2. Effect of xanthine/xanthine oxidase on t-PA mRNA levels.** Northern blot analysis of total cellular RNA isolated following a 2-h incubation period from HUVE cells exposed to control (A), xanthine/xanthine oxidase (B), and a mixture of xanthine/xanthine oxidase and superoxide dismutase/catalase (C). Samples were probed for t-PA and β-actin mRNA. The molecular size, expressed in kilobases (kb) was determined using the RNA calibration mixture. For details, see "Materials and Methods."

### TABLE III

**Effect of oxidant scavenger enzymes on t-PA antigen secretion in HUVE cells**

|          | Without SOD/CAT | With SOD | With CAT | With SOD/CAT | With inactivated SOD/CAT |
|----------|-----------------|----------|----------|--------------|-------------------------|
| Control  | 4.0 ± 1.2       | 4.0 ± 1.2| 4.0 ± 1.2| 4.0 ± 1.2    | 4.0 ± 1.2               |
| X + XO   | 8.5 ± 1.3       | 6.3 ± 0.4| 5.6 ± 0.5| 4.2 ± 1.3    | 8.1 ± 1.2               |

### TABLE IV

**Effect of oxidant scavengers on t-PA and PAI-1 mRNA levels induced with xanthine/xanthine oxidase in HUVE cells**

The data are from a single experiment, representative or three individual experiments with n = 3. SOD, superoxide dismutase; CAT, catalase; X, xanthine; XO, xanthine oxidase.

| Incubation time | t-PA mRNA | PAI-1 mRNA |
|-----------------|-----------|------------|
|                 | Control   | X/XO and SOD + CAT | Control   | X/XO and SOD + CAT |
| h               |           |             |           |                     |
| 2               | 0.6 ± 0.1 | 1.8 ± 0.5   | 0.4 ± 0.3 | 100 ± 20            |
| 8               | 12 ± 3.8  | 20 ± 2.1    | 14 ± 1.1  | 81 ± 44             |
| 24              | 6.9 ± 1.6 | 17 ± 0.5    | 9.8 ± 2.5 | 300 ± 50            |

Hybridization with the β-actin 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 oxidants or scavengers.

### DISCUSSION

Regulation of intravascular fibrinolysis is partly obtained at the level of the endothelial cell, a significant site of synthesis of circulating t-PA and PAI-1 (37). Derangements leading to endothelial cell injury can cause cellular dysfunction resulting in abnormal hemostasis and in thrombotic and hemorrhagic disorders in man (38-40). A number of factors have been reported to affect both t-PA and PAI-1 activity in cultured endothelium.

The present study demonstrates that cultured human vascular endothelial cells respond to a brief, sublethal extracellular oxidant perturbation. A 15-min exposure of HUVE cells to the xanthine/xanthine oxidase mixture caused a profibrinolytic response consisting of increased t-PA synthesis and secretion without affecting the inhibitor PAI-1. In addition, an increase of t-PA mRNA was observed that was suggestive of enhanced transcription.

The exogenous addition of the oxygen radical scavengers superoxide dismutase/catalase in combination ablated the increase in t-PA synthesis and secretion as well as mRNA, confirming that the endothelial cell profibrinolytic response is specifically mediated via extracellular oxidant generation.

Oxygen-free radicals are generated in cells and tissues under a variety of conditions and thus mediate many pathologic processes. They induce injury to cells by oxidizing proteins, initiating lipid peroxidation, inactivating enzymes, etc. (41). Yet, neither the magnitude nor mechanism(s) of target cell injury is well-defined.

Endothelial cells located on the luminal surface of the vascular wall are vulnerable to attack by reactive oxygen species released by activated phagocytes as they are recruited to sites of injury. It is unclear to what extent nonlethal oxidant exposure generated by cell or cell-free systems may interfere with overall endothelial cell function(s). Cell lysis and death occur following long term exposure (42), whereas shorter treatments have led to disruption of several functions: plasma membrane organization (43), shape and transport (44), hydrolysis of inositol phospholipids (45), and impaired low density lipoprotein receptor-mediated endocytosis (46). We have earlier described alterations in platelet adherence and prostacyclin release from xanthine/xanthine oxidase-treated endothelium (47).

Although the xanthine/xanthine oxidase system has been extensively used to monitor the biologic impact of oxidant metabolites generated in the extracellular space, this model may be somewhat limited in providing direct information on
the effects of phagocyte-derived oxidants. Xanthine/xanthine oxidase systems produce $H_2O_2$ or $OH^-$ from $O_2$ and $H_2O$. Phagocytic cells, however, do not ordinarily permit released $H_2O_2$ to reach substantial concentrations and thus generate very little hydroxyl radical (48). This is in contrast to the xanthine/xanthine oxidase system which permits the accumulation of generated $H_2O_2$ enabling more complex interactions between $O_2$, transition metals, and $H_2O_2$ to proceed. Thus caution is mandatory when extrapolating extracellular effects mediated by cell-free systems to more physiologic sources of oxidants in vivo. The use of a well-defined soluble generating system for oxygen radicals has several advantages which include the quantitation of dose response, neutralization, and/or amelioration with appropriate enzymes and scavengers as well as defined kinetics of onset and duration of effect.

Whether the observed profibrinolytic response of human endothelium following extracellular oxidant targeting actually occurs in vivo remains speculative. This phenomenon may be an acute physiological response to external stimulation rather than a result of pathologic injury and remains to be further investigated.

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