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Thrombin stimulates increased plasminogen activator inhibitor-1 release from liver compared to lung endothelium

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ABSTRACT

Background: Plasminogen activator inhibitor-1 (PAI-1) is a major regulator of the fibrinolytic system, covalently binding to tissue plasminogen activator and blocking its activity. Fibrinolysis shutdown is evident in the majority of severely injured patients in the first 24 h and is thought to be due to PAI-1. The source of this PAI-1 is thought to be predominantly endothelial cells, but there are known organ-specific differences, with higher levels thought to be in the liver. Thrombin generation is also elevated in injured patients and is a potent stimulus for PAI-1 release in human umbilical endothelial cells. We hypothesize that thrombin induces liver endothelial cells to release increased amounts of PAI-1, *versus* pulmonary endothelium, consisting of both stored PAI-1 and a larger contribution from *de novo* PAI-1 synthesis.

Methods: Human liver sinusoidal endothelial cells (LSECs) and human microvascular lung endothelial cells (HMVECs) were stimulated *in vitro* ± thrombin (1 and 5 IU/mL) for 15-240 min, the supernatants were collected, and PAI-1 was measured by enzyme-linked immunosorbent assays. To elucidate the PAI-1 contribution from storage *versus de novo* synthesis, cycloheximide (10 μg/mL) was added before thrombin in separate experiments.

Results: While both LSECs and HMVECs rapidly stimulated PAI-1 release, LSECs released more PAI-1 than HMVECs in response to high-dose thrombin, whereas low-dose thrombin did not provoke immediate release. LSECs continued to release PAI-1 over the ensuing 240 min, whereas HMVECs did not. Cycloheximide did not inhibit early PAI-1 release from LSECs but did at the later time points (30-240 min).

Conclusions: Thrombin elicits increased amounts of PAI-1 release from liver endothelium compared with lung, with a small presynthesized stored contribution and a later, larger increase in PAI-1 release via *de novo* synthesis. This study suggests that the liver may be an

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important therapeutic target for inhibition of the hypercoagulable surgical patient and the associated complications that result.

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Introduction

The serpin plasminogen activator inhibitor-1 (PAI-1) is a major regulator of fibrinolysis, covalently binding to tissue plasminogen activator (tPA) and blocking activation of plasminogen.[1] Elevated PAI-1 levels have been noted in numerous pathologic conditions, including thrombi causing acute coronary syndrome as well as circulating in the plasma of trauma and intensive care unit (ICU) patients.[2-4] In these patients, this circulating PAI-1 elevation is associated with increased mortality.[2,5] There is also evidence to suspect that it is a main determinant of fibrinolysis shutdown in trauma patients, associated with increased mortality due to multiorgan failure.[6-8]

PAI-1 is synthesized in a multitude of cell types including endothelial cells, macrophages, hepatocytes, smooth muscle cells, and platelets.[9-12] Furthermore, there is a wide variance in the quantity released from different organs and similar cell lines, for example, endothelium, in those organs. Postmortem tissue samples demonstrated variable levels of PAI-1 immunoreactivity, with the highest total concentrations and activity consistently found in the liver and significantly lower levels in the lung.[9] In addition, while liver transplant patients exhibit significant increases in tPA activity during the hepatectomy portion of the liver transplant (and decreases in PAI-1 activity), reperfusion of the donor liver leads to significant increases in PAI-1 and decreases in tPA activity.[13] The potential unique contributions from different organ-specific vascular beds are relatively unexplored with most of the literature focused on human umbilical endothelial cells.[14-17] The majority of *in vitro* endothelial work is done with human umbilical endothelial cells and extrapolated to all endothelium; however, the presence of unique organ-specific endothelial bed differences in PAI-1 production calls to question the potential for differences in other properties of organ-specific endothelium, including permeability, protein synthesis, and response to ischemic insults.

Thrombin is a serine protease known to be elevated in the plasma of injured patients with both procoagulant and anticoagulant properties.[18-20] Importantly, thrombin provokes PAI-1 release in murine and human endothelial cells.[16] Incorporating previous work showing high activity and total levels of PAI-1 in the liver and the effects of liver reperfusion during transplant,[9,13] we hypothesize that liver endothelium are capable of increased PAI-1 release with thrombin stimulation (compared to lung endothelium) with a small component of presynthesized stored PAI-1 and a larger contribution from *de novo* PAI-1 synthesis.

Materials and methods

Materials

All reagents were purchased from Sigma Chemical Company (St. Louis, MO) unless otherwise stated. Thrombin was obtained from Enzo Life Sciences, Inc. (Farmingdale, NY). Human total PAI-1 enzyme-linked immunosorbent assay (ELISA) kits were purchased from Molecular Innovations (Novi, MI).

Methods

Endothelial cells

Human liver sinusoidal endothelial cells (LSECs) and human lung microvascular endothelial cells (HMVECs) were purchased from Lonza (Allendale, NJ). Cells were grown to 80%-90% confluence, and all experiments were done in 12-well plates. In order to elucidate the PAI-1 contribution from storage *versus de novo* protein synthesis, cycloheximide (10 μg/mL) was added to select wells for 30 min at 37°C before thrombin stimulation. Media were then removed and replaced with Krebs-ringer-phosphate with dextrose (KRPD). High (5 IU/mL) and low dose (1 IU/mL) thrombin, selected based on previous experiments,[16,21] or KRPD (controls) were added for 5, 15, 30, 120, and 240 min at 37°C and 5% CO_2. Time intervals were chosen based on pilot studies demonstrating optimal conditions for the short-active half-life of thrombin (1 min *in vivo,* 3-5 min *ex vivo*) and minimal disruption of cell count and adherence in the presence of KRPD media. Cell survival and adherence were confirmed with visualization under the microscope at the end of the experiment. The supernatants were removed, aliquotted, flash frozen, and stored at −80°C for future use.

Enzyme-linked immunosorbent assays

Total PAI-1 release was measured in the supernatants from LSECs or HMVECs by ELISA according to manufacturer recommendations.

Statistical analysis

The data are shown as the mean ± the standard error of the mean. Statistical analysis was completed using SPSS, version 24 (IBM). Low-dose thrombin was compared with high-dose thrombin at each time point, and LSECs were compared with HMVECs at each time point in a separate hypothesis. As this was a prespecified hypothesis driven protocol, statistical significance was defined as $P < 0.05$ on a two-tailed paired parametric *t*-test.[22,23]

Results

High-dose thrombin provokes immediate PAI-1 release and low-dose thrombin does not

To determine optimum thrombin concentration for stimulation of PAI-1 release, human LSECs were exposed to low- (1 IU/mL) and high-dose (5 IU/mL) thrombin for 15-240 min. High-dose thrombin stimulated about 2.5-fold increase (over control) in PAI-1 release at the early time points (15 and 30 min; $P = 0.01$ and 0.04, respectively), whereas low-dose thrombin

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