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Thrombin Stimulates Increased PAI-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 (tPA) and blocking its activity. Fibrinolysis shutdown is evident in the majority of severely injured patients in the first 24 hours 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 HUVECs. 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, 5IU/ml) for 15-240 minutes, the supernatants were collected and PAI-1 was measured by ELISA. To elucidate the PAI-1 contribution from storage versus *de novo* synthesis, cycloheximide (10ug/ml) was added prior to 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, while low dose thrombin did not provoke immediate release. LSECs continued to release PAI-1 over the ensuing 240 minutes,

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while HMVECs did not. Cycloheximide did not inhibit early PAI-1 release from LSECs but did at the later time points (30-240 minutes).

Conclusion—Thrombin elicits increased amounts of PAI-1 release from liver endothelium compared to lung, with a small presynthesized stored contribution and a later, larger increase in PAI-1 release via *de novo* synthesis. This study suggests the liver may be an important therapeutic target for inhibition of the hypercoagulable surgical patient and the associated complications that result.

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 ICU patients.²⁻⁴ In these patients, this circulating PAI-1 elevation is associated with increased mortality.²⁵ There is also evidence to suspect 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, e.g. endothelium, in those organs. Post-mortem 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 t-PA 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 t-PA 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 (HUVECs).¹⁴⁻¹⁷ The majority of *in vitro* endothelial work is done with HUVECs 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 (Farmingdale, NY). Human total PAI-1 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 minutes at 37°C prior to thrombin stimulation. Media was then removed and replaced with Krebs-ringer-phosphate with dextrose (KRPD). High (5IU/ml) and low dose (1IU/ml) thrombin, selected based on previous experiments,¹⁶²¹ or KRPD (controls) were added for 5, 15, 30, 120, and 240 minutes at 37°C and 5% CO₂. Time intervals were chosen based on pilot studies demonstrating optimal conditions for the short active half-life of thrombin (1 minute *in vivo*, 3-5 minutes *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 (ELISAs)—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 to high dose thrombin at each time point, and LSECs were compared to HMVECs at each time point in a separate hypothesis. As this was a pre-specified hypothesis driven protocol, statistical significance was defined as p<0.05 on a 2-tailed paired parametric t-test.
2223

Results

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

To determine optimum thrombin concentration for stimulation of PAI-1 release, human liver sinusoidal endothelial cells were exposed to low dose (1IU/ml) and high dose (5IU/ml) thrombin for 15-240 minutes. High dose thrombin stimulated about 2.5-fold increase (over control) in PAI-1 release at the early time points (15, 30m; p=0.01, 0.04 respectively) while low dose thrombin did not demonstrate increased PAI-1 release compared to control (Figure 1). This increased to 4-fold at the later time points (120, 240m; p=0.025, 0.021) in the high dose thrombin, while low dose thrombin demonstrated about 2-fold increase at the late time points, although not statistically significant (Figure 1).

Thrombin stimulates rapid PAI-1 release from liver endothelium and, to a lesser extent, lung endothelium

To investigate whether endothelial cells *in vitro* release PAI-1, human liver sinusoidal endothelial cells (LSECs) and human pulmonary microvascular endothelial cells (HMVECs) were stimulated with high dose thrombin (5U/ml). As demonstrated previously, thrombin-stimulated LSECs generated ~2-4-fold increase in PAI-1 release as compared to controls at 15-240 minutes (Figure 2), while thrombin-stimulated HMVECs demonstrated less significant increases in PAI-1 at 15 and 30 minutes but not at 120 and 240 minutes (Figure 2). Both types of endothelial cells released PAI-1 at 15 minutes versus controls (change PAI-1 relative to control: LSECs 6.7ng/ml, $p=0.015$; HMVECs 3.55ng/ml, $p<0.001$). Thrombin-stimulated LSECs continued to release significantly more PAI-1 at 30, 120, and 240 minutes versus controls (30m- 7.92ng/ml, $p<0.001$; 11.86ng/ml, $p=0.019$, 19.4ng/ml, $p=0.019$ respectively), while HMVEC PAI-1 release approached that of controls (HMVECs 30m- 2.54ng/ml, $p=0.009$; 0.13ng/ml, $p=0.063$; 0.867, $p=0.090$) (Figure 2). Thrombin-stimulated liver endothelium released significantly more PAI-1 at all time-points as compared to lung endothelium (15m- $p=0.002$, 30m- $p=0.008$, 120m- $p=0.008$, 240m- $p=0.025$) (Figure 2).

High dose thrombin provokes both stored release of PAI-1 and new PAI-1 synthesis from LSECs

LSECs were further studied to determine whether these endothelial cells contain stored PAI-1, which is rapidly released upon stimulation. LSECs were pretreated with cycloheximide (10ug/ml), a protein translation inhibitor, followed by thrombin stimulation (5IU/ml). Cycloheximide had no effect on early PAI-1 release after thrombin stimulation versus controls (5, 15 minutes), whereas it did effect late PAI-1 release (30, 120 minutes) with that difference becoming statistically significant at 120 minutes (Figure 3, $p=0.009$). Cycloheximide halted further PAI-1 release after 30 minutes.

Discussion

PAI-1 is a major regulator of fibrinolysis, with high levels correlated to increased morbidity and mortality in trauma, ICU, and septic patients.²⁵ In addition, our group and others have recently characterized a fibrinolysis shutdown trauma-induced-coagulopathy, with an increased mortality due to multi-organ failure, with PAI-1 thought to play a major role.⁶⁷²⁴ While endothelium are thought to play a large part in the production of PAI-1, the organ-specific contributions are unknown. We have shown that, while liver and lung endothelium are capable of producing PAI-1, liver endothelium is the dominant source. In addition, there exists a small amount of presynthesized stored PAI-1 available for immediate release, but the majority is synthesized and then released following activation.

Currently, there is little knowledge about the contributions of plasma PAI-1 in the setting of trauma and sepsis, especially early in the disease process. Our data demonstrating increased PAI-1 synthesis in hepatic endothelium is consistent with previous literature where high levels of PAI-1 immunoreactivity were found in postmortem organ staining in the liver while low-levels were found in the lung tissue.⁹ Our data also provides further information on the

source of the increased PAI-1 activity seen during the reperfusion phase of a donor liver.¹³ Previous work on organ specific endothelial release is limited. Kumar *et al* compared different endothelial cells and the release of PAI-1 and demonstrated increased PAI-1 immunoreactivity in pulmonary artery endothelium versus iliac vein endothelium in response to TNF α stimulation.²⁵ While it is well-known that TNF α levels increase in trauma and ICU patients,²⁶ this experiment used two endothelial cell types which may not have large contributions to the circulating PAI-1 concentration.

The disparate contributions of PAI-1 from different endothelial beds to the circulation may be physiologically advantageous as pulmonary hemorrhage would be life threatening such that the rapid release of PAI-1 may limit such a hemorrhage. However, continued high PAI-1 release in the lung would lead to microvascular occlusion resulting in decreased gas exchange, further contributing to decreased tissue oxygenation during shock. Conversely, the liver is very susceptible to hemorrhagic injury as the result of trauma and is a significant producer of procoagulant proteins. Thus, the immediate and sustained release of high-levels of PAI-1 could be beneficial in continuing to limit life-threatening hepatic hemorrhage. Up to this point, the majority of *in vitro* endothelial work has been done in human umbilical vein endothelial cells, including function, permeability, and protein synthesis. Our findings suggest that not all endothelial cells function the same and further research should be tailored to the organ of interest. In addition, future research designed to limit the amount of PAI-1 produced leading to hypercoagulable complications in surgery and specifically liver transplant and trauma patients should focus on liver endothelium and other strong PAI-1 contributors.

There are several limitations in the current study. First, the *in vitro* measurement of total PAI-1 levels rather than PAI-1 activity as this environment lacks the necessary cofactors for stabilization of an active PAI-1 protease (such as vitronectin).^{27,28} Also, while endothelium are thought to be the most significant contributor to PAI-1 release,³ further research is needed into other cell types (platelets, macrophages, neutrophils) to determine their roles in the release of PAI-1 into the circulation.

Conclusion

Thrombin provokes varied amounts of PAI-1 from organ specific endothelium, with higher release from liver endothelium. While there is a component of pre-synthesized stored PAI-1 in endothelium, the majority is synthesized and released upon stimulation.

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Figure 1. High Dose Thrombin provokes rapid and sustained PAI-1 release

High dose thrombin (5IU/ml) provoked immediate release of PAI-1 from LSECs, while low dose thrombin (1IU/ml) did not (15 and 30 minutes). At the later time points (120, 240 minutes), high dose thrombin provoked even larger increases in PAI-1 release from LSECs (about 4-fold), while low dose thrombin demonstrated about 2-fold increase compared to controls, although not statistically significant ($p=0.052$, 0.213 , respectively). * $p<0.05$ compared to control at that time point. $n=3-4$.

Figure 2. Thrombin-stimulated LSECs release more PAI-1 as compared to HMVECs
Liver sinusoidal endothelium is capable of releasing significantly more PAI-1 at 15, 30, and 120 minutes as compared to lung endothelium. $n=4-5$, $*p<0.05$ as compared to lung endothelium at the same time point. $\#p<0.05$ compared to control HMVECs at the same time point.

Figure 3. Thrombin-stimulated hepatic endothelium releases pre-synthesized PAI-1

The ability of LSECs to release presynthesized stored PAI-1 was explored with pretreatment with cycloheximide (10ug/ml), a protein translation inhibitor. Cycloheximide did not inhibit early PAI-1 release (5, 15 minutes) from LSECs but did inhibit late PAI-1 release (30, 120 minutes) showing early PAI-1 release is from pre-synthesized stored PAI-1, while the late time points are reflective of new PAI-1 synthesis (inhibited by cycloheximide). n=3, *p<0.04 as compared to thrombin with cycloheximide at the same time point.