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Scott B Swarbreck

Dan Secor

Christopher G Ellis

Michael D Sharpe

John X Wilson

See next page for additional authors

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Authors

Scott B Swarbreck, Dan Secor, Christopher G Ellis, Michael D Sharpe, John X Wilson, and Karel Tymł

Effect of ascorbate on plasminogen activator inhibitor-1 expression and release from platelets and endothelial cells in an in-vitro model of sepsis

Scott B. Swarbreck^{a,b,c}, Dan Secor^{a,b}, Christopher G. Ellis^{a,c},
Michael D. Sharpe^d, John X. Wilson^e and Karel Tym^{a,b,c}

The microcirculation during sepsis fails due to capillary plugging involving microthrombosis. We demonstrated that intravenous injection of ascorbate reduces this plugging, but the mechanism of this beneficial effect remains unclear. We hypothesize that ascorbate inhibits the release of the antifibrinolytic plasminogen activator inhibitor-1 (PAI-1) from endothelial cells and platelets during sepsis.

Microvascular endothelial cells and platelets were isolated from mice. Cells were cultured and stimulated with lipopolysaccharide (LPS), tumor necrosis factor alpha (TNF α), or thrombin (agents of sepsis), with/without ascorbate for 1–24 h. PAI-1 mRNA was determined by quantitative PCR. PAI-1 protein release into the culture medium was measured by ELISA. In platelets, PAI-1 release was measured after LPS, TNF α , or thrombin stimulation, with/without ascorbate. In endothelial cells, LPS and TNF α increased PAI-1 mRNA after 6–24 h, but no increase in PAI-1 release was observed; ascorbate did not affect these responses. In platelets, thrombin, but not LPS or TNF α , increased PAI-1 release; ascorbate inhibited this increase at low extracellular pH. In unstimulated endothelial cells and

platelets, PAI-1 is released into the extracellular space. Thrombin increases this release from platelets; ascorbate inhibits it pH-dependently. The data suggest that ascorbate promotes fibrinolysis in the microvasculature under acidotic conditions in sepsis. *Blood Coagul Fibrinolysis* 26:000–000
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^aCritical Illness Research, Lawson Health Research Institute, ^bDepartment of Physiology and Pharmacology, ^cDepartment of Medical Biophysics, ^dDepartment of Anesthesia and Perioperative Medicine, Critical Care Western, University of Western Ontario, London, Ontario, Canada and ^eDepartment of Exercise and Nutrition Sciences, University at Buffalo, Buffalo, New York, USA

Correspondence to Dr Karel Tym, Critical Illness Research, Victoria Research Laboratories, 6th Floor, 800 Commissioners Road East, London, ON N6C 2V5, Canada
Tel: +1 519 685 8500 ext 55076; e-mail: ktym@lhsc.on.ca

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Introduction

Sepsis – a systemic inflammatory response to an infection – leads to numerous physiological abnormalities, including a fibrinolytic abnormality due to elevated plasma levels of plasminogen activator inhibitor-1 (PAI-1) [1–3]. PAI-1 can inhibit the plasmin-mediated dissolving of microthrombi in the microvasculature and thus contribute to disseminated intravascular coagulation. To this end, we have shown that sepsis plugs the capillary bed, involving increased occurrence of both fibrin plaques and platelet adhesion/aggregation in capillaries [4–6]. To our knowledge, the role of PAI-1 in sepsis-induced capillary plugging is not known.

We have reported that, in septic mice, an intravenous injection of ascorbate delayed 6 h after the onset of sepsis unplugs capillaries [6]. Although numerous mechanisms could explain this beneficial effect of ascorbate [6], it is possible that ascorbate enhances the dissolving of capillary microthrombi by inhibiting PAI-1. Oxidative stress has been implicated in the increase in both mRNA and protein expression of PAI-1 in endothelial cells [7].

Because endothelial cells and platelets (i.e. cells in the milieu of capillary microthrombi) can release PAI-1 into

the extracellular space [8–10], the objective of the present study was to use simple in-vitro and ex-vivo models of sepsis to examine whether ascorbate affects PAI-1 expression and release from these two cell types. We hypothesized that ascorbate reduces the expression and release of PAI-1 from endothelial cells and platelets in sepsis.

Methods

Animal preparation

All experiments were conducted under protocols approved by the University of Western Ontario Council on Animal Care. Endothelial cells for in-vitro experiments were obtained from male wild-type C57BL/6 mice (age 1.5–3 months) from Charles River Laboratories (Sherbrooke, Quebec, Canada). Preliminary experiments indicated no sex-specific differences in platelet function; therefore platelets for ex-vivo experiments were obtained from male or female wild-type mice of the same strain. Mice were anesthetized with ketamine (80 mg/kg) and xylazine (4 mg/kg) prior to cell harvest or platelet collection. All supplies were purchased from Sigma–Aldrich (Oakville, Ontario, Canada) unless otherwise stated.

In-vitro model of sepsis in cultured endothelial cells

Endothelial cells were harvested from the hind limb skeletal muscle as previously described [11,12]. We have chosen this tissue because sepsis-induced capillary plugging and the beneficial effect of ascorbate against this plugging have been observed in the mouse skeletal muscle [5,6]. Briefly, the muscle was excised and digested in a collagenase solution for 30 min. The endothelial cells were purified using a lectin-coated magnetic bead immunoseparation. The cells were cultured and used for experiments up to passage 10. For each experiment, cells were grown to confluence and treated with ascorbate (100 $\mu\text{mol/l}$) for 1, 3, 6, or 24 h, concurrently with either lipopolysaccharide (LPS) (10 $\mu\text{g/ml}$) or tumor necrosis factor alpha (TNF α) (10 ng/ml). Alternatively, cells were treated with thrombin (0.0375 U/ml) for 3–5 min at the end of the 24-h period. We used LPS and TNF α as conventional agents of sepsis as these substances have been shown to initiate endotoxemia *in vivo* [13,14]. The chosen dose of ascorbate results in physiological levels of intracellular ascorbate [15]. Thrombin (0.0375 U/ml) has been used to activate platelets to mimic sepsis *ex vivo* [16]. We have used this concentration of thrombin in endothelial cells as an alternative in-vitro model of sepsis. After completion of experiments, cell supernatants were collected and frozen, and the cells were lysed in Tris-NaCl-Triton-EDTA (TNTE) buffer or Trizol (Invitrogen, Burlington, Ontario, Canada).

Ex-vivo model of sepsis in isolated platelets

Blood was collected (9:1) in acid citrate dextrose solution, and platelets were isolated as detailed previously [16]. Briefly, the blood was centrifuged at 179 relative centrifugal force (rcf), and the supernatant was retained and centrifuged at 774 rcf to pellet the platelets. The platelet pellet was resuspended in Dulbecco's phosphate-buffered saline (DPBS), divided evenly among the control and treatment groups on the particular experimental day, and stimulated with thrombin (0.0375 U/ml), LPS (10 $\mu\text{g/ml}$), or TNF α (10 ng/ml) with or without ascorbate (100, 1000, or 10 000 $\mu\text{mol/l}$). In some experiments with ascorbate, we used sodium hydroxide (NaOH) to adjust the pH of the DPBS + ascorbate solution to 7.4. In other experiments, we exposed platelets to DPBS solution, whose pH was set to 3.8, 5.8, or 7.0 by hydrochloric acid (HCl). The platelets were incubated at 37 °C for 10 min, then centrifuged to pellet the platelets. The supernatant was retained and frozen, and the platelet pellet homogenized in TNTE buffer or Trizol.

Plasminogen activator inhibitor-1 mRNA expression

The mRNA was isolated using the phenol–chloroform extraction method. The isolated mRNA was purified using DNase I and Reverse Transcribed using Superscript II (Invitrogen). The mRNA was quantified using Quantifast SYBR Green (Qiagen, Mississauga, Ontario,

Canada) as directed on a Bio-Rad CFX96 thermal cycler (Bio-Rad, Mississauga, Ontario, Canada). The primers used to detect the mRNA are as follows: PAI-1 (forward: 5'-GACAGCACTGTCAGGGTCCATAG-3' and reverse: 5'-GCGGTCCTCCTTCACAAAGCTC-3') and β -actin as a housekeeping gene (forward: 5'-TCGTGGCCGCTCTAGGCACCA-3' and reverse: 5'-GTTGGCCTTAGGGTTCAGGGGGG-3'). The mRNA was amplified using the following cycling protocol: 95 °C for 5 min and then 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 80 °C for 30 s.

Plasminogen activator inhibitor-1 protein expression and release

Both intracellular PAI-1 protein (i.e. PAI-1 in cell lysates) and PAI-1 release (i.e. PAI-1 in cell supernatants) were quantified using the total PAI-1 ELISA kit (IMPAIKT-TOT; Innovative Research, Novi, Michigan, USA) according to the manufacturer's directions.

Statistical analysis

Data are shown as mean \pm standard error (SE). A minimum of five different cell lines were used for the endothelial cell experiments. A minimum of three groups of pooled platelets were used for the platelet experiments. Statistical analysis was performed using a repeated-measures one-way analysis of variance (ANOVA) and the Bonferroni multiple comparison test. Results are significant with a *P* value less than 0.05.

Results

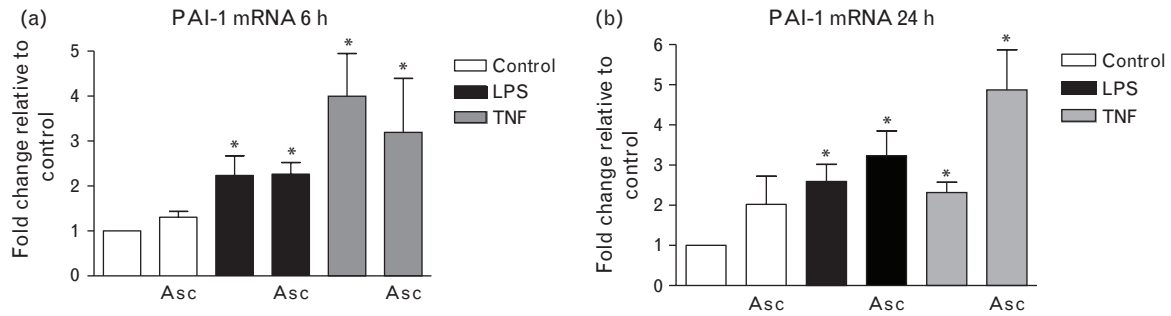
Endothelial cell plasminogen activator inhibitor-1 mRNA and protein expression

mRNA analysis of endothelial cell PAI-1 expression revealed that LPS and TNF α raised expression after 6 and 24 h of stimulation (Fig. 1), but not after 1 and 3 h of stimulation (data not shown), when compared with control unstimulated cells. Ascorbate had no effect on the expression of PAI-1 mRNA at 1–6 h, but there was a tendency for increased PAI-1 mRNA in control and TNF α groups at 24 h. Interestingly, the LPS or TNF α -induced increase in mRNA seen at 6 and 24 h did not translate into increased production of PAI-1 protein at 24 h (Fig. 2). Surprisingly, in control cells, ascorbate increased the production of PAI-1 protein. In LPS or TNF α -treated cells, ascorbate had no effect on intracellular PAI-1 protein (Fig. 2).

Plasminogen activator inhibitor-1 protein release from endothelial cells

For all control, LPS, and TNF α groups at 24 h, PAI-1 protein release was much larger than the amount of PAI-1 protein remaining in the cells (Fig. 2). Ascorbate did not affect the PAI-1 release in any of the groups (Fig. 2). Figure 3 shows the effect of acute stimulation of endothelial cells with thrombin. Ascorbate did not affect the PAI-1 production or release from control cells. Thrombin

Fig. 1



Increases in PAI-1 mRNA expression after 6 and 24 h of endotoxin treatment were not altered by ascorbate (Asc). Isolated endothelial cells were treated with either LPS (10 μ g/ml) or TNF α (10 ng/ml) for 6 or 24 h as indicated. Some cells were also treated with 100 μ mol/l Asc concurrently. Total cell mRNA was extracted, reverse-transcribed, and PAI-1 mRNA was quantified using qPCR. At 6 (a) and 24 h (b), there was a significant increase in PAI-1 expression with both LPS and TNF α treatment. Asc did not appear to have an effect on this increase in PAI-1 expression. (*) $P < 0.05$ compared to control ($n = 4-7$ different cell lines per treatment). LPS, lipopolysaccharide; PAI-1, plasminogen activator inhibitor-1; qPCR, quantitative PCR; TNF α , tumor necrosis factor alpha.

had no effect on intra-endothelial PAI-1 protein in cells treated with either vehicle or ascorbate for 24 h, nor on PAI-1 protein release from these cells (Fig. 3). Thrombin did not affect the increased intracellular PAI-1 in control cells treated with ascorbate (Fig. 3).

Plasminogen activator inhibitor-1 protein release from platelets

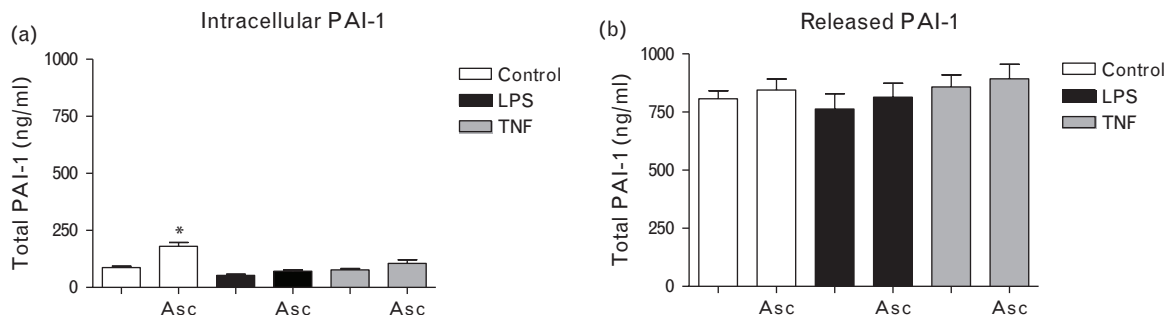
There was experiment-to-experiment variability in the number of platelets collected. We tested randomly selected aliquots of platelets to determine the platelet counts. The mean number of platelets added per treatment (100 μ l) was $29.9 \times 10^6 \pm 3.9 \times 10^6$ ($n = 10$). In general, this variability resulted in different levels of intracellular and released PAI-1 protein in control platelets among experiments. Because of this variability, the results from treated and control platelets were paired for each experiment.

Figure 4 shows that neither LPS nor TNF α affected intracellular and released PAI-1 protein levels when compared to the control unstimulated platelets. We then tested whether thrombin would initiate platelet PAI-1 release. As each treatment group had both control and thrombin only treatments, we pooled these results and observed that the platelets contained 0.38 ± 0.06 ng/ml of PAI-1 intracellularly and spontaneously released 0.11 ± 0.01 ng/ml PAI-1. Upon stimulation with thrombin, release of PAI-1 from the platelets significantly increased with 0.29 ± 0.05 ng/ml remaining intracellularly and 0.32 ± 0.06 ng/ml released ($P < 0.05$ compared to control, $n = 21$ per group).

Effect of pH and ascorbate on platelet plasminogen activator inhibitor-1 protein release

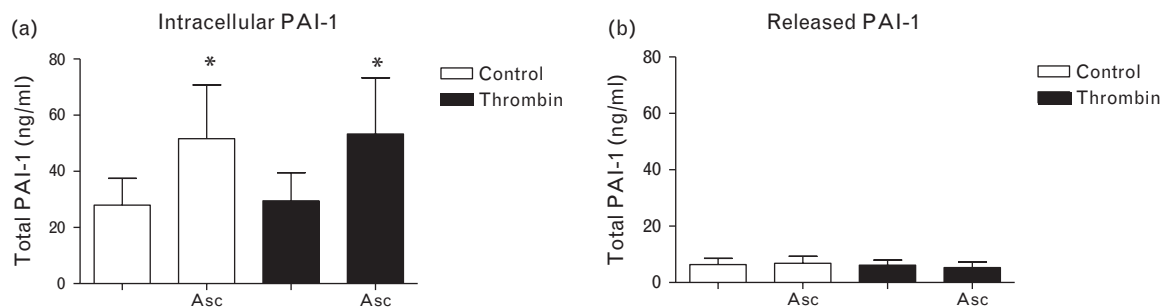
As thrombin-induced platelet PAI-1 release, our next objective was to determine whether ascorbate affected

Fig. 2



Lipopolysaccharide (LPS) or TNF α did not alter the PAI-1 production or release in endothelial cells. Endothelial cells were treated with LPS (10 μ g/ml) or TNF α (10 ng/ml) for 24 h, the supernatant was collected, and the cells were lysed. The PAI-1 content in the cells and released in the supernatant was quantified using an ELISA. Ascorbate (Asc) treatment alone for 24 h significantly increased the PAI-1 production in the endothelial cells (a). Co-treatment with either LPS or TNF α eliminated the observed increase in PAI-1 protein. No treatment altered the PAI-1 release into the supernatant (b). (*) $P < 0.05$ compared to control ($n = 4$ cell lines per group). PAI-1, plasminogen activator inhibitor-1; TNF α , tumor necrosis factor alpha.

Fig. 3



Short-term thrombin has no effect on the release of PAI-1 protein from endothelial cells. Endothelial cells previously treated with ascorbate (Asc) or fresh media for 24 h were treated with thrombin (0.0375 U/ml) for 5 min, then the supernatant was collected and the cells lysed. Thrombin had no effect on the release of PAI-1 into the supernatant (b). Ascorbate treatment significantly increased the PAI-1 production intracellularly (a). (*) $P < 0.05$ compared to control ($n = 5$ cell lines per group). PAI-1, plasminogen activator inhibitor-1.

this release. Using a 100 $\mu\text{mol/l}$ concentration of ascorbate, there was no effect on platelet concentration or release of PAI-1 (Fig. 5a and b); however, using a dose of 1000 $\mu\text{mol/l}$, ascorbate partially inhibited its release (Fig. 5c and d). While a 100 $\mu\text{mol/l}$ concentration of ascorbate had no effect on the extracellular pH, ascorbate at 1000 $\mu\text{mol/l}$ reduced the pH to 7.0. In a parallel experiment, we prepared DPBS solution in which the pH was set to 7.0 by HCl. In this experiment, the thrombin-induced platelet PAI-1 release was inhibited; this inhibition was similar to that of ascorbate at 1000 $\mu\text{mol/l}$ (Fig. 5c and d). Ascorbate administered at a concentration of 10 000 $\mu\text{mol/l}$ (pH of DPBS + ascorbate solution reduced to 3.8) potently inhibited the thrombin-induced platelet PAI-1 release (Fig. 5e and f). To test whether this inhibition was pH-sensitive, we adjusted the pH of the DPBS + ascorbate solution

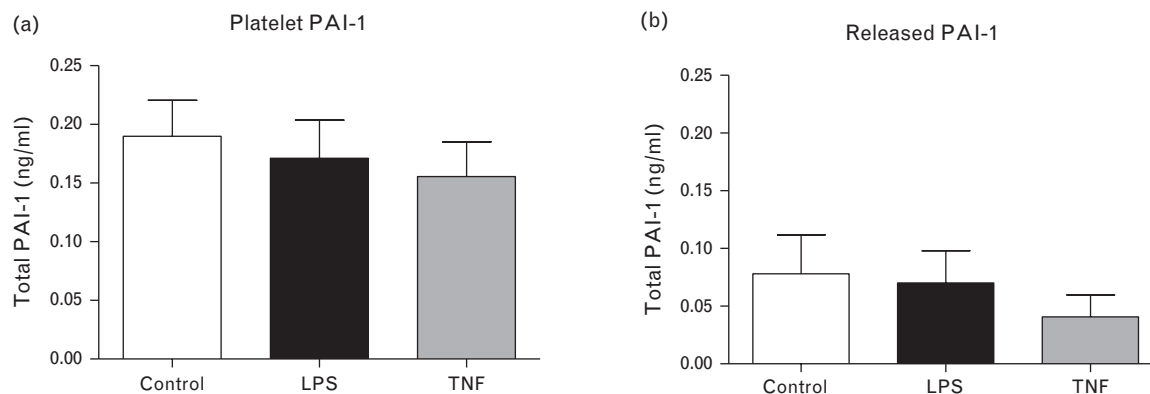
to 7.4 using NaOH. Under these conditions, the inhibitory effect of ascorbate was abolished (PAI-1 released by thrombin alone: $181.2 \pm 33.2\%$ of release by control platelets; PAI-1 released by thrombin + ascorbate: $202.5 \pm 19.5\%$ of control, $n = 4$ per group, $P = 0.60$). In the parallel experiment (pH set to 3.8 by HCl), the platelet PAI-1 release was similarly inhibited as by 10 000 $\mu\text{mol/l}$ ascorbate (Fig. 5e and f).

Because of the similarity between the effects of ascorbate and HCl, we tested whether the inhibition of thrombin-induced PAI-1 release from platelets was pH-dependent. Figure 6 shows this dependency.

Discussion

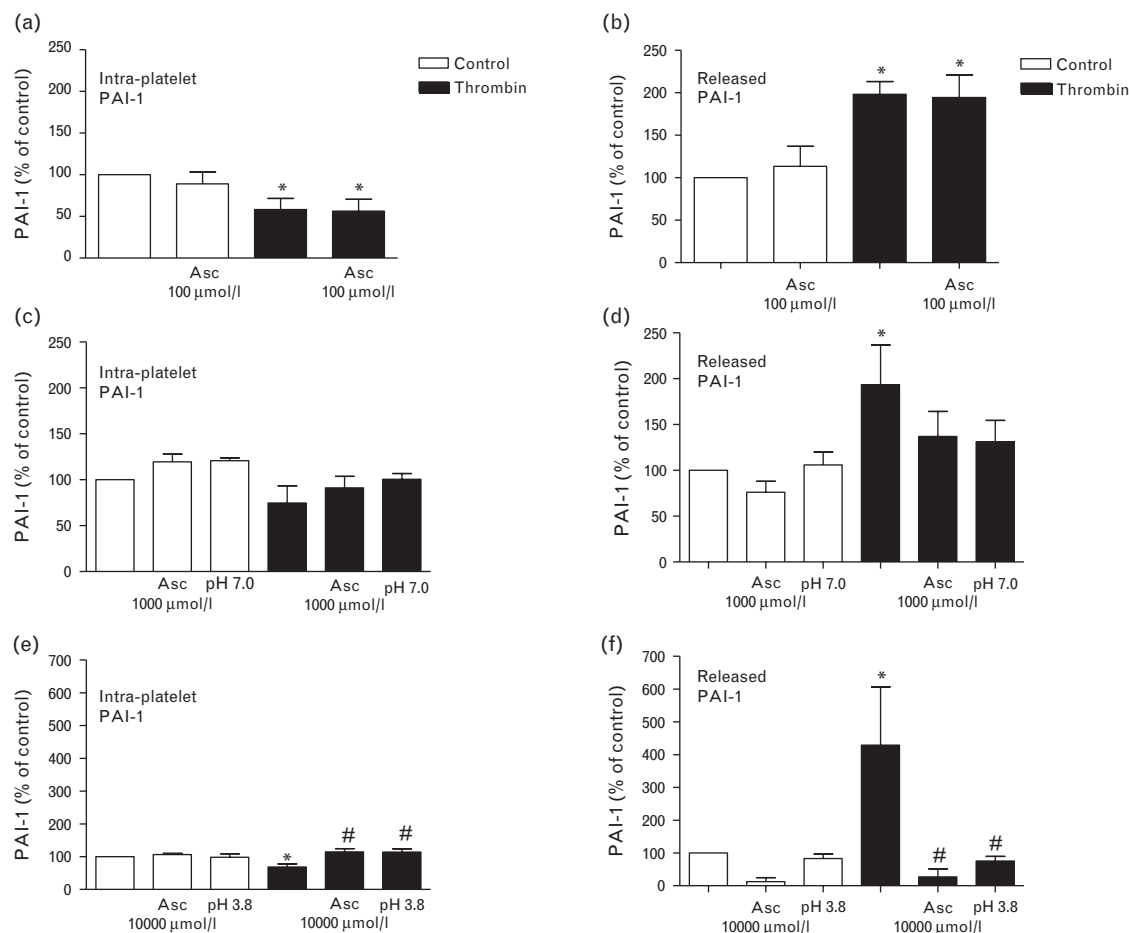
We previously showed that an intravenous bolus of ascorbate reduces capillary plugging, leading to improved

Fig. 4



Plasminogen activator inhibitor-1 (PAI-1) release by platelets is not induced by LPS or $\text{TNF}\alpha$. Platelets were isolated from whole mouse blood, stimulated with LPS (10 $\mu\text{g/ml}$) or $\text{TNF}\alpha$ (10 ng/ml) for 10 min, then the supernatant and platelet pellets were collected and subjected to ELISA. Neither LPS nor $\text{TNF}\alpha$ treatment affected intraplatelet PAI-1 protein expression (a) or caused release of PAI-1 protein from the platelets into the supernatant (b) ($n = 6$ platelet isolations per group). LPS, lipopolysaccharide; PAI-1, plasminogen activator inhibitor-1; $\text{TNF}\alpha$, tumor necrosis factor alpha.

Fig. 5



Effects of ascorbate (Asc) and pH on thrombin-induced PAI-1 release from platelets. Isolated platelets were treated with ascorbate (100 $\mu\text{mol/l}$, panels a and b; 1000 $\mu\text{mol/l}$, panels c and d; or 10 000 $\mu\text{mol/l}$, panels e and f) for 3 min and then by thrombin (0.0375 U/ml) for 10 min. The supernatant and platelet pellets were collected and subjected to ELISA. Thrombin induced a significant release of PAI-1 from the platelets into the supernatant (b, d, and f). Ascorbate at 1000 or 10 000 $\mu\text{mol/l}$ prevented the thrombin-induced PAI-1 release (d and f). Panels c–e and f also show results from experiments in which we used HCl to set the pH of extracellular fluid to 7.0 or 3.8 (i.e. bars labeled by pH 7.0 or pH 3.8). (*) $P < 0.05$ compared to control unstimulated platelets, (#) $P < 0.05$ compared to thrombin-stimulated platelets [$n = 4$ platelet isolations per group (a, b), $n = 4$ platelet isolations per group (c, d), and $n = 3–6$ platelet isolations per group (e, f)]. PAI-1, plasminogen activator inhibitor-1.

survival in sepsis [4–6]. The present study used simplified in-vitro and ex-vivo models of sepsis to examine the effect of ascorbate on PAI-1 production and release from endothelial cells and platelets, that is, cells found in the milieu of developing capillary microthrombi. We show that PAI-1 production and release from endothelial cells and platelets is not affected by a low-dose ascorbate (100 $\mu\text{mol/l}$). However, increasing concentrations of ascorbate may inhibit PAI-1 release from platelets in a pH-dependent manner.

Plasminogen activator inhibitor-1 mRNA expression in endothelial cells

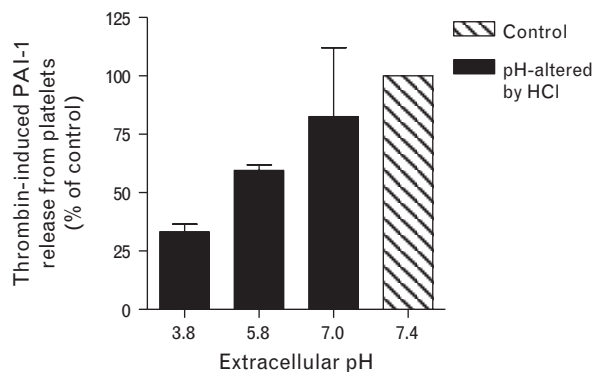
Our finding that 1 or 3 h LPS/TNF α does not alter endothelial cell PAI-1 mRNA contrasts with the reported early PAI-1 increase after LPS treatment [17]. However, the increased PAI-1 mRNA observed at 6 and 24 h mirrors

that of the previous studies [17,18]. It is possible that interspecies differences between mouse and human cells may be responsible for the delayed response to LPS. Indeed, it has been reported that humans have a markedly increased sensitivity to LPS compared to mice, and that interspecies differences in sensitivity to infections can have profound effects on survival [13,19]. Ascorbate did not appear to have any effect on PAI-1 mRNA expression after 1–6 h of treatment. At 24 h, ascorbate tended to increase the PAI-1 mRNA expression in the control cells and in TNF α -treated cells (Fig. 1b).

Plasminogen activator inhibitor-1 protein expression and release from endothelial cells

Because the amount of PAI-1 released from endothelial cells was much larger than PAI-1 remaining in the cells (Fig. 2), it appears that these cells contribute significantly

Fig. 6



Thrombin-induced release of PAI-1 from platelets is pH-dependent. Isolated platelets were treated with thrombin (0.0375 U/ml) for 10 min in Dulbecco's phosphate-buffered saline (DPBS, control bar), or in DPBS solution, the pH of which was set to 3.8, 5.8, or 7.0 by HCl. The thrombin-induced PAI-1 release was reduced with lowered pH ($n=3-4$ platelet isolations per group). HCl, hydrochloric acid; PAI-1, plasminogen activator inhibitor-1.

to the total PAI-1 within the vasculature. LPS, TNF α , and thrombin did not affect intra-endothelial and released PAI-1 protein (Figs. 2 and 3). This lack of effect contrasts with previous studies in human umbilical vein endothelial cells, in which significant increases in PAI-1 protein production were observed [17,18]. Given the interspecies variability, it is possible that the expression of PAI-1 in mouse cells differs from that of the human cells.

Effect of ascorbate on plasminogen activator inhibitor-1 protein expression in endothelial cells

The tendency of ascorbate to increase PAI-1 mRNA in control cells translated to increased PAI-1 protein production in these cells. LPS and TNF α inhibited this increased production (Fig. 2a). However, increased intra-endothelial PAI-1 did not result in increased PAI-1 release into the extracellular space (Fig. 2b). The mechanism of this phenomenon is not clear. We speculate that ascorbate may increase the stability of PAI-1, thus enhancing the total content [20].

Overall, the long period required for ascorbate to induce any changes in PAI-1 content in endothelial cells (e.g. 24h) does not lend itself to explain any acute in-vivo effects of ascorbate on fibrinolysis [4-6]. Thus, in the septic microvasculature, restoring capillary blood flow over the short period of 1h following ascorbate administration [6] may not be explained by ascorbate's inhibition of PAI-1 release from endothelial cells.

Effect of ascorbate and pH on plasminogen activator inhibitor-1 release from platelets

Consistent with our report that LPS and TNF α do not affect platelet aggregation [16], LPS and TNF α did not affect PAI-1 release from platelets (Fig. 4). This finding

agrees with the reports of lack of effect of LPS [21] and TNF α [22] in platelets isolated from mice, though others have observed spontaneous platelet activation upon LPS stimulation [23]. We note that these conflicting studies were conducted with platelets from species other than mice, suggesting that interspecies differences may be involved in the different responses. It is also possible that neither LPS nor TNF α directly stimulates platelets, but rather induces the production of other signaling molecules (i.e. thrombin) that ultimately activate platelets [24]. The present ex-vivo model may lack the cells necessary for the production of these signaling molecules.

The observed PAI-1 release from platelets following short-term stimulation with thrombin is consistent with the literature [9,10]. Ascorbate at a concentration of 100 μ mol/l did not alter the release of PAI-1 from platelets. This is in contrast to our previous report that ascorbate reduces the rate of thrombin-induced aggregation [16]. It is possible that we were unable to observe a reduction in the rate of PAI-1 release due to our inability to measure the PAI-1 content in real time.

Treatment with 1000 and 10 000 μ mol/l ascorbate at an unbuffered pH 7.0 and 3.8, respectively, inhibited the thrombin-induced platelet PAI-1 release (Fig. 5). Critically ill patients can develop severe acidosis, when the blood pH drops below 6.8 [25], and this level may be as low as 6.0 in localized regions, particularly during ischemia [26]. Thus, pH values in patients fall within the range of pH in the present study. Importantly, when the pH of ascorbate solution (10 000 μ mol/l) was adjusted to 7.4, the inhibition of PAI-1 release by ascorbate was abolished, indicating the inhibition is pH-dependent. Consistently, we demonstrated that the thrombin-induced platelet PAI-1 release can be inhibited by lowering the pH of the extracellular solution (Fig. 6). Our finding of a potential pH-dependent effect of ascorbate agrees with the report that thrombin-induced platelet adhesion is reduced by approximately 25% at pH 7.0 and 50% at pH 6.5 [26].

Could the reduced plugging in septic capillaries by ascorbate injection be partially explained in terms of the pH-dependent inhibition of PAI-1 release from platelets? Our preliminary experiments in sham mice injected with ascorbate (10 mg/kg) demonstrated a decreased blood pH from 7.2 to 7.0 at 5 min postinjection. At this pH, our observed inhibition of thrombin-induced PAI-1 release by ascorbate may just begin to be effective (Fig. 5d). When ascorbate is used at a higher dose (e.g. 200 mg/kg [27,28]), this effectiveness may increase. Considering that blood pH during sepsis may be acidotic, we speculate that treatment of septic mice with ascorbate may further lower the pH, resulting in inhibition of PAI-1 release by ascorbate in a pH-dependent manner.

In conclusion, we have confirmed the presence and release of PAI-1 protein by both endothelial cells and

platelets – two cell types in close proximity to a developing intravascular thrombus during sepsis. Although ascorbate did not directly affect thrombin-induced PAI-1 release from platelets, it reduced the release indirectly through a pH-dependent mechanism.

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Conflicts of interest

There are no conflicts of interest.

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