Ascorbate inhibits platelet-endothelial adhesion in an in-vitro model of sepsis via reduced endothelial surface P-selectin expression

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Plugging of the capillary bed can lead to organ failure and mortality in sepsis. We have reported that intravenous ascorbate injection reduces platelet adhesion to the capillary wall and capillary plugging in septic mice. Both platelet adhesion and capillary plugging require P-selectin, a key adhesion molecule. To elucidate the beneficial effect of ascorbate, we hypothesized that ascorbate reduces platelet-endothelial adhesion by reducing P-selectin surface expression in endothelial cells. We used mouse platelets, and monolayers of cultured microvascular endothelial cells (mouse skeletal muscle origin) stimulated with lipopolysaccharide, to examine platelet-endothelial adhesion. P-selectin mRNA expression in endothelial cells was determined by real-time PCR and P-selectin protein expression at the surface of these cells by immunofluorescence. Secretion of von Willebrand factor from cells into the supernatant (a measure of P-selectin-containing granule exocytosis) was determined by ELISA. Lipopolysaccharide (10 μg/ml, 1 h) increased platelet-endothelial adhesion. P-selectin-blocking antibody inhibited this adhesion. Lipopolysaccharide also increased P-selectin mRNA in endothelial cells, P-selectin expression at the endothelial surface, and von Willebrand factor secretion. Ascorbate pretreatment (100 μmol/l, 4 h) inhibited the increased platelet adhesion, surface expression of P-selectin, and von Willebrand factor secretion, but not the increase in P-selectin mRNA. The lipopolysaccharide-induced increase in platelet-endothelial adhesion requires P-selectin presence at the endothelial surface. Ascorbate's ability to reduce this presence could be important in reducing both platelet adhesion to the capillary wall and capillary plugging in sepsis. *Blood Coagul Fibrinolysis* 28:28–33 Copyright © 2017 Wolters Kluwer Health, Inc. All rights reserved.

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Introduction

Sepsis is the tenth leading cause of mortality in the United States, [1] with a mortality rate near 40% [2]. Circulatory dysfunction, including cessation of blood flow in septic capillaries, can lead to multiple organ failure [3,4]. Capillary blood flow impairment is associated with increased platelet adhesion and fibrin deposition in capillaries, suggesting that microthrombi have formed, plugging the capillary bed [5]. Increased platelet trapping in this bed could explain the reduced platelet count in plasma of septic patients, which correlates with increased mortality [6]. The cessation of capillary blood flow increases the diffusional distance for oxygen to reach cell mitochondria, leading to tissue hypoxia and organ failure [7,8].

Microvascular thrombosis and disseminated intravascular coagulation (DIC, [9]) contribute to the severity of sepsis. During sepsis, increased platelet-endothelial adhesion may be critical to the capillary flow impairment [5]. P-selectin is a key platelet-endothelium adhesion molecule [10] because inhibition of P-selectin results in decreased platelet adhesion in the septic capillary, as well as in reduced cessation of blood flow [5]. P-selectin is stored in Weibel-Palade bodies in endothelial cells and, upon stimulation, it is expressed at the surface of the cell where it facilitates platelet adhesion through binding its counter receptor P-selectin glycoprotein ligand-1 (PSGL1) [11]. Reactive oxygen species (ROS) generated during sepsis have also been implicated in the impairment of capillary blood flow [12,13]. ROS have been shown to enhance activation of platelets [14] and endothelial cells [15].

Ascorbate has been investigated as a possible treatment for sepsis [16,17]. An intravenous bolus of ascorbate has been shown to prevent and reverse the cessation of blood flow in septic capillaries as well as increase survival of septic mice [18,19]. Sepsis-induced increases in adhesion of platelets and their aggregates in capillaries were also inhibited by ascorbate treatment [5]. Although several mechanisms have been proposed to explain the beneficial

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effect of ascorbate in the septic microvasculature [13], the precise mechanism of this effect is unclear.

Our recent study showed that ascorbate could reduce platelet adhesion during sepsis, as well as improve capillary blood flow [5]. However, it was not possible to determine whether ascorbate inhibited platelet-endothelial adhesion directly, or indirectly, via hemodynamic effects of ascorbate on capillary blood flow. To address this issue, the present study employed a simple in-vitro platelet-endothelial cell adhesion assay. This in-vitro model could determine the effect of sepsis on the endothelial P-selectin expression and the effect of ascorbate on this expression. We hypothesized that sepsis-induced platelet-endothelial adhesion and endothelial P-selectin genomic expression and surface expression are reduced by ascorbate.

Methods

Reagents

Lipopolysaccharide (LPS), calcein-AM, protease inhibitor cocktail, Superscript II, and ascorbate were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). The fluorescein isothiocyanate (FITC)-conjugated P-selectin antibody (clone: Wug.E9) was purchased from Emfret Analytics (Eibelstadt, Germany). P-selectin-blocking antibody (clone: RB40.34) and rat IgG1 lambda isotype control were purchased from BD Pharmingen (Mississauga, Ontario, Canada). TRIZOL and Hoechst 33342 nuclear stain were purchased from Invitrogen (Burlington, Ontario, Canada). A horseradish peroxidase (HRP) tagged antirat IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania USA). An HRP antirabbit IgG antibody was purchased from Cell Signalling Technology (Danvers, Massachusetts, USA). Quantifast SYBR Green master mix was purchased from Qiagen (Mississauga, Ontario, Canada).

Endothelial cell harvest and culture

All experimental protocols were approved by the University of Western Ontario Council on Animal Care. Skeletal muscle microvascular endothelial cells were harvested from wild-type (C57BL6) mice and isolated as performed previously in our laboratory [20,21]. Briefly, skeletal muscle from the hindlimb of mice was collected and digested. The digest was plated and grown to confluence. Endothelial cells were isolated by lectin coated magnetic beads. Endothelial phenotype was confirmed by the presence of von Willebrand factor VIII as previously described [22]. To model sepsis, endothelial cells were treated with LPS (10 μg/ml) for 1 h. Ascorbate (100 μmol/l) was applied to cells as a pretreatment 4 h prior to LPS treatment. In some experiments, a P-selectin blocking antibody (clone: RB40.34, 1 : 1000) or control IgG was added to endothelial cells concurrently with LPS.

Platelet collection and isolation

Blood (600 μl) was collected from anaesthetized male mice via carotid artery puncture into a vial containing 100 μl of acid citrate dextrose solution. Platelets were isolated by the method described by Singer *et al.* [23]. Briefly, platelet-rich plasma was collected by centrifuging the whole blood at 120*g* for 8 min. The plasma was then re-spun to remove any collected leukocytes. Finally, the platelets were pelleted at 735*g* for 10 min and resuspended in phosphate buffer solution (PBS).

In-vitro adhesion model to mimic the platelet-endothelial cell interaction

To visualize platelets with fluorescence microscopy, isolated platelets were incubated with 8 μmol/l calcein-AM for 10 min. The platelets were pelleted at 735*g* for 10 min to remove excess calcein-AM and re-suspended in fresh PBS. Following endothelial cell treatments, $\sim 3 \times 10^6$ platelets were added to the confluent monolayer of $\sim 0.25 \times 10^6$ endothelial cells and co-incubated under static conditions for 1 h, and then washed. The remaining platelets adhering to the endothelial cells were visualized with a Zeiss fluorescence microscope using a 20× magnification objective. Labeled platelets were then counted in the entire area of the microscopic field of view (0.43 mm × 0.32 mm). Each treatment group was done in triplicate and five arbitrary fields of view (chosen blindly) were used per replicate. Counts of attached platelets were repeated by a separate individual blinded to the conditions.

P-selectin and PSGL1 mRNA expression in cultured endothelial cells

P-selectin and PSGL1 mRNA were measured by real-time qPCR. After treatment, total mRNA from the endothelial cells was collected using TRIZOL according to the manufacturer's instructions. mRNA was then reversed transcribed to cDNA by Superscript II. We used qPCR to determine the mRNA expression levels of P-selectin (primers: forward 5'-GTCCACGGAGAGTTTGGTGT-3' and reverse 5'-AAGTGGTGTTCGGACCAAAG-3') and PSGL1 (primers: forward 5'-CTTC CTTGTGCTGCTGACCAT-3' and reverse 5'-TCAG GGTCCTCAAAATCGTCATC-3') and used β-actin (primers: forward 5'-TCGTGGGCCGCTCTAGGCACCA-3' and reverse 5'-GTTGGCCTTAGGGTTCAGGGGGG-3') as our reference gene. The qPCR was carried out using Quantifast SYBR Green master mix on a MiniOpticon Real-Time PCR System (Bio-Rad, Mississauga, Ontario, Canada). We used 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. SYBR green fluorescence intensity was acquired at 80°C in each amplification cycle. Subsequently, a melt curve was generated, starting at 60°C and taking measurements every 0.2°C until 95°C was reached.

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Immunofluorescence for P-selectin surface expression

P-selectin surface expression was measured by immunofluorescence. Endothelial cells treated as described previously were incubated with a FITC-conjugated P-selectin antibody (clone:Wug.E9, 1 : 200) concurrently with control or LPS treatment. The cells were then washed thoroughly and fixed with 4% paraformaldehyde. Following fixing, cells were co-stained with Hoechst 33342. The cells were imaged with a Zeiss Observer D1 microscope using AxioVision Rel 4.7 software. Cell counts were taken from a minimum of 5 fields of view chosen at random, per experimental group. Cells were defined as P-selectin positive if the fluorescence intensity was greater than that of unlabeled cells (i.e. cells that were not incubated with the antibody).

Von Willebrand factor ELISA

P-selectin is contained together with von Willebrand factor (vWF) in Weibel-Palade granules under the surface of endothelial cells. Exocytosis of these granules delivers to the surface both vWF and P-selectin [11]. Endothelial cells grown to confluence were treated with dialyzed serum medium with or without ascorbate (100 μmol/l) for 4 h. The medium was replaced by fresh medium without ascorbate and the cells were treated with LPS (10 μg/ml) for 1 h. The cell supernatant was collected and measured for vWF using a commercially available ELISA kit (Cusabio, Wuhan, China).

Statistical analysis

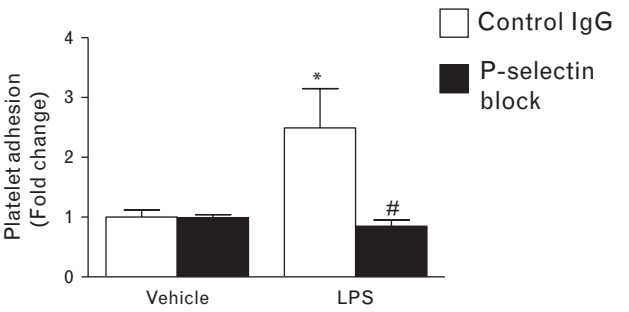
Data are represented as mean ± standard error and n represents the number of mice or separate cell culture experiments. The cells were harvested from at least three different mice per treatment group. Data were analyzed by one-way ANOVA followed by t test with Bonferroni correction for multiple comparisons. P values less than 0.05 were considered significant.

Results

P-selectin-blocking antibody and ascorbate inhibit LPS-induced platelet-endothelial cell adhesion

To determine whether the previously reported inhibition by ascorbate of platelet-endothelial interactions in capillary *in vivo* [5] was a direct effect of ascorbate on endothelial function, rather than an indirect effect on capillary hemodynamics, an in-vitro model was employed in the present study. In this model, LPS increased platelet-endothelial cell adhesion under static conditions (Figs. 1 and 2). The LPS-induced increase in adhesion was prevented by addition of a P-selectin-blocking antibody applied to endothelial cells concurrently with LPS (Fig. 1). These data indicate that P-selectin is necessary for the LPS-induced platelet adhesion. Pretreatment of endothelial cells with ascorbate for 4 h inhibited the LPS-induced increase in platelet-endothelial cell adhesion, (Fig. 2) indicating that ascorbate plays a direct role in reducing platelet-endothelial interactions.

Fig. 1

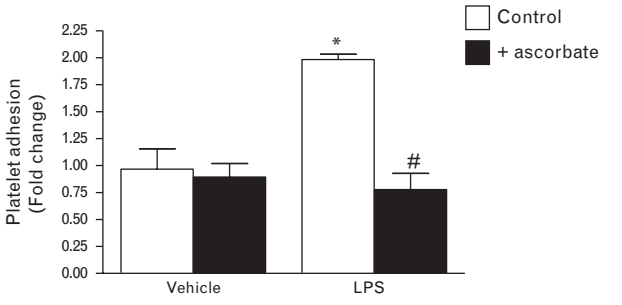


Effect of P-selectin-blocking antibody on lipopolysaccharide (LPS)-induced endothelial cell-platelet adhesion. Microvascular endothelial cells (mouse hindlimb muscle origin) were treated with LPS (10 μg/ml) for 1 h and then washed. Immediately thereafter, cells were co-incubated with untreated platelets for 1 h, and then washed again (prior to incubation, platelets were labeled with fluorescent calcein AM). Platelets adhering to the endothelial surface were counted by fluorescence microscopy. A separate group of endothelial cells was co-treated with a P-selectin-blocking antibody during the LPS treatment. LPS significantly increased the endothelial cell-platelet adhesion. P-selectin block inhibited this increased adhesion. (*significantly different from control vehicle-treated group, #significantly different from control LPS-treated group, $P < 0.05$, $n = 5$/group).

Lipopolysaccharide increases P-selectin mRNA but not PSGL1 mRNA expression

In endothelial cells, LPS treatment significantly increased P-selectin mRNA expression (Fig. 3). Pretreatment of the endothelial cells with ascorbate did not affect this increase (Fig. 3). Thus, ascorbate did not affect the *de novo* synthesis of P-selectin. Furthermore, LPS did not affect the counter-receptor PSGL1 mRNA expression in endothelial cells (Fig. 3).

Fig. 2



Effect of ascorbate on LPS-induced endothelial cell-platelet adhesion. Endothelial cells treated with LPS for 1 h were co-incubated with untreated platelets for 1 h, washed, and adherent platelets were counted by fluorescence microscopy. A separate group of cells was pretreated for 4 h with ascorbate (100 μmol/l) prior to LPS treatment. LPS treatments significantly increased the endothelial cell-platelet adhesion. Ascorbate inhibited this increased adhesion. (*significantly different from control vehicle-treated group, #significantly different from control LPS-treated group, $P < 0.05$, $n = 5-8$/group).

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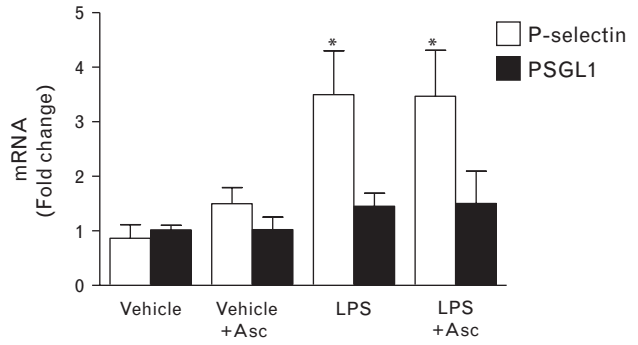
Fig. 3

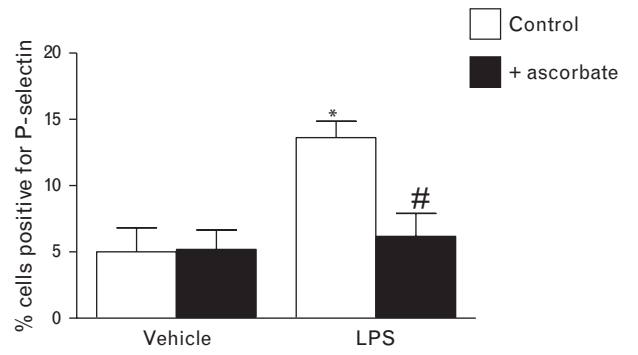


Effect of ascorbate on P-selectin and P-selectin glycoprotein ligand-1 (PSGL1) mRNA expression in LPS-treated endothelial cells. Endothelial cells treated with LPS for 1 h were assayed for P-selectin or PSGL1 mRNA expression by real-time qPCR. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS treatment. LPS treatments significantly increased P-selectin but not PSGL1 mRNA. Ascorbate did not affect this increased expression. (*significantly different from control vehicle-treated group, $P < 0.05$, n = 6–7/group).

Ascorbate inhibits lipopolysaccharide-induced P-selectin surface expression in endothelial cells

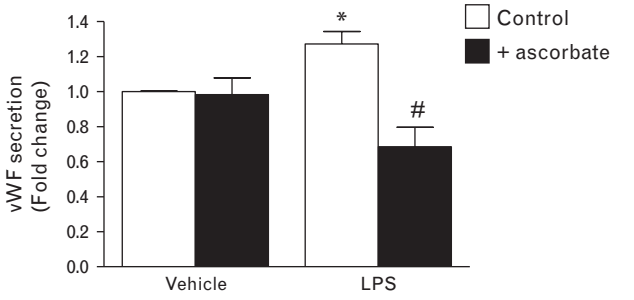
Although ascorbate did not affect expression of P-selectin mRNA, ascorbate could alter P-selectin expression functionally, in terms of its protein expression at the endothelial surface. Based on immunofluorescence probing for P-selectin protein expression at the endothelial surface, we observed that LPS significantly increased the number of cells expressing P-selectin (Fig. 4). Ascorbate pretreatment inhibited this increase (Fig. 4).

Fig. 4



Effect of ascorbate on LPS-induced P-selectin surface expression. Endothelial cells treated with LPS for 1 h with or without a pretreatment with ascorbate for 4 h were assayed for cell surface P-selectin protein by immunofluorescence. LPS significantly increased the cell surface P-selectin expression. Ascorbate inhibited this increased expression. (*significantly different from control vehicle-treated group, #significantly different from control LPS-treated group, $P < 0.01$, $n = 4–6$/group).

Fig. 5



Effect of ascorbate on LPS-induced release of von Willebrand factor (vWF) from endothelial cells. Cell culture medium of endothelial cells treated with LPS for 1 h was collected and assayed for vWF by ELISA. A separate group of cells was pretreated for 4 h with ascorbate prior to LPS treatment. LPS increased vWF release from endothelial cells. Ascorbate inhibited this increased vWF release. (*significantly different from control vehicle-treated group, #significantly different from control LPS-treated group, $P < 0.05$, $n = 5$/group).

Ascorbate inhibits vWF release in lipopolysaccharide-treated endothelial cells

To examine mechanistically how ascorbate affects P-selectin surface expression, we measured vWF release from endothelial cells into the supernatant. Both P-selectin and vWF are contained in Weibel-Palade granules under the surface of endothelial cells and ascorbate could potentially affect exocytosis of these granules [11]. LPS increased vWF release from endothelial cells and ascorbate inhibited this increase (Fig. 5).

Discussion

In the present study, LPS treatment of endothelial cells increased platelet-endothelial adhesion. P-selectin-blocking antibody and ascorbate inhibited this increase. LPS increased P-selectin mRNA and surface protein expression in endothelial cells, as well as vWF release from these cells. Ascorbate inhibited the increases in adhesion, surface P-selectin, and vWF release, but not the increase in P-selectin mRNA. It appears that ascorbate affected P-selectin functionally to inhibit LPS-induced platelet-endothelial adhesion.

Sepsis was reported to induce platelet adhesion to the capillary wall *in vivo* [5]. Our present in-vitro model successfully mimicked this observation. LPS has been shown to increase platelet-endothelial cell adhesion [24]. In our model, increased adhesion was P-selectin-dependent, as blockage of P-selectin via an antibody prevented stimulated adhesion. As only endothelial cells received treatments in the present model, activation of endothelial cells was responsible for changes in platelet-endothelial adhesion. Therefore, we propose that LPS causes an increase in P-selectin expression in the endothelial cells, leading to increased adhesion. The observed LPS-induced increases in P-selectin mRNA (Fig. 3)

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and P-selectin protein surface expression (Fig. 4) are consistent with this proposal (similar LPS-induced increases have been seen elsewhere [25–28]). As P-selectin-PSGL1 binding can produce further signaling that also leads to adhesion involving other adhesion proteins [10], the role of P-selectin in the observed adhesion could be direct or indirect.

We previously reported that ascorbate reduces platelet adhesion in septic capillaries [5]. To our knowledge, we show here for the first time that ascorbate directly inhibits platelet-endothelial adhesion in an in-vitro assay. Previous reports have shown that other antioxidants, namely vitamin E and quercetin, can reduce platelet-endothelial adhesion *in vitro* [29,30]. Szuwart *et al*. found that the inhibitory effect of vitamin E on platelet adhesion was seen in platelet-rich plasma but not in isolated platelets [30].

Treatment with ascorbate did not affect P-selectin mRNA expression in the endothelial cells (Fig. 3). This finding contradicts a previous report that the antioxidant nebivolol inhibits P-selectin mRNA expression in human macrovascular endothelial cells stimulated with oxidative low-density lipoprotein [31]. The difference could be explained by the use of a different stimuli, species, or cell type. However, importantly, we show for the first time that pretreatment of the endothelial cells with ascorbate inhibited the LPS-induced increase in P-selectin protein surface expression. Together with the P-selectin-dependent platelet adhesion shown in Figure 1, this indicates that ascorbate reduces platelet-endothelial interaction by reducing P-selectin on the surface of the endothelial cells. Others have reported similar effects with antioxidants in terms of reducing P-selectin surface expression caused by thrombin [32] or hypoxia/re-oxygenation [33] in human umbilical vein endothelial cells. Thus, it is possible that the antioxidant effect of ascorbate is responsible for the inhibition of LPS-induced platelet-endothelial adhesion and that this inhibition is mediated via reduced P-selectin surface expression on endothelial cells.

It is unlikely that over the period of 1 h of LPS treatment, the de-novo synthesis of P-selectin protein was increased. The observed increase in surface P-selectin expression was more likely because of exocytosis of P-selectin protein already contained in Weibel-Palade granules (WPG). To address this possibility, we measured vWF secretion as a marker of WPG exocytosis. Again, consistent with the literature [34], LPS increased vWF release from endothelial cells (Fig. 5). We also demonstrated that ascorbate pretreatment inhibits vWF release from LPS-stimulated endothelial cells. A similar inhibitory effect of the antioxidant genipin on vWF release was reported for thrombin-stimulated cells [35]. It is therefore likely that the inhibition of P-selectin surface expression by ascorbate was through reduced WPG exocytosis.

In conclusion, we used an *in vitro* model of sepsis in mice to demonstrate that LPS increases platelet-endothelial adhesion P-selectin dependently. Consistent with the inhibitory effect of ascorbate against this adhesion observed in septic capillaries *in vivo*, ascorbate also inhibited this adhesion in the present in-vitro model. Ascorbate also inhibited LPS-induced increases in endothelial P-selectin surface expression and in vWF release, suggesting that the inhibition involves posttranslational modification of P-selecting function at the endothelial surface.

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Conflicts of interest

There are no conflicts of interest.

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