Intracellular Ascorbate Prevents Endothelial Barrier Permeabilization by Thrombin*

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*Running title: Ascorbate prevents endothelial permeabilization by thrombin

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Background: Vitamin C (ascorbate) promotes endothelial barrier stability during inflammation. Results: Ascorbate prevents inflammatory decreases in cAMP. Conclusion: cAMP preserves the cortical actin cytoskeleton and prevents stress fiber formation. Significance: Dietary vitamin C may promote vascular integrity in patients with inflammatory diseases.

ABSTRACT

Intracellular ascorbate (vitamin C) has previously been shown to tighten the endothelial barrier and maintain barrier integrity during acute inflammation in vitro. However, the downstream effectors of ascorbate in the regulation of endothelial permeability remain unclear. In this study, we evaluated ascorbate as a mediator of thrombin-induced barrier permeabilization in human umbilical vein endothelial cells (HUVEC) and their immortalized hybridoma line, Ea.hy926. We found that the vitamin fully prevented increased permeability to the polysaccharide inulin by thrombin in a dose-dependent manner, and it took effect both before and after subjection to thrombin. Thrombin exposure consumed intracellular ascorbate but not the endogenous antioxidant GSH. Likewise, the antioxidants dithiothreitol and Tempol did not reverse permeabilization. We identified a novel role for ascorbate in preserving cAMP during thrombin stimulation, resulting in two downstream effects. First, ascorbate maintained the cortical actin cytoskeleton in a Rap1- and Rac1-dependent manner, thus preserving stable adherens junctions between adjacent cells. Second, ascorbate prevented actin polymerization and formation of stress fibers by reducing the activation of RhoA and phosphorylation of myosin light chain. While ascorbate and thrombin both required calcium for their respective effects, ascorbate did not prevent thrombin permeabilization by obstructing calcium influx. However, preservation of cAMP by ascorbate was found to depend on both the production of nitric oxide by endothelial nitric oxide synthase, which ascorbate is known to activate, and the subsequent generation cGMP by guanylate cyclase. Together, these data implicate ascorbate in the prevention of inflammatory endothelial barrier permeabilization and explain the underlying signaling mechanism.

In vascular beds lacking significant fenestrations, the endothelium is the most significant barrier to the transfer of large serum proteins and other molecules from blood to the interstitium. Adherens junctions between cells contribute significantly to this barrier function by providing intercellular adhesion (1). These transmembrane protein junctions are stabilized by their intracellular association with the cortical actin cytoskeleton. During inflammation, the permeability of this barrier is increased due to formation of gaps between endothelial cells. These gaps are caused by the opening of adherens junctions and the centripetal tightening of the actin cytoskeleton. This opening of adherens junctions is caused by the actin polymerization and formation of stress fibers that are driven by the RhoA/Rac1/myosin light chain pathway.
cytoskeleton, which pulls the cells apart (2,3). It follows that many inflammatory mediators including thrombin, histamine and various oxidants compromise barrier integrity by diminishing cortical actin and promoting cytoskeletal rearrangement to form contractile actin stress fibers (2,4). In contrast, barrier-stabilizing mediators such as cAMP, nitric oxide (NO) (5) and the small GTPases Rap1 and Rac1 (2) enhance adherens junctions and preserve cortical actin.

Increasing intracellular ascorbate (vitamin C) concentrations to physiologic levels both tightens the unstimulated endothelial barrier (6,7) and prevents inflammatory permeabilization of this barrier (8-10). Tightening of the unstimulated endothelial permeability barrier by ascorbate required function of endothelial nitric oxide synthase (eNOS) and guanylate cyclase (11), suggesting that ascorbate may tighten the endothelial barrier by increasing intracellular NO and cGMP. However, the downstream signaling mechanisms following stimulation by inflammatory agents have yet to be evaluated.

In this work we examined whether and how intracellular ascorbate can prevent thrombin-induced endothelial permeabilization in human umbilical vein endothelial cells (HUVEC) and in a hybridoma cell line derived from HUVEC (Ea.hy926). We found that intracellular ascorbate at what are likely physiologic intracellular concentrations prevented thrombin-induced increases in endothelial barrier permeability by preserving both cAMP and cortical actin in an NO-dependent manner.

MATERIALS AND METHODS

Reagents – Sigma-Aldrich Chemical Co. (St. Louis, MO) supplied ascorbate, 1,2-Bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tetrakis(acetoxymethyl ester) (BAPTA-AM), dehydroascorbic acid (DHA), dithiothreitol (DTT), N-2-hydroxyethylpiperazine N′-2-ethanesulfonic acid (Hepes), 4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl (4-hydroxy-TEMPO) (Tempol), Nω-nitro-L-arginine methyl ester hydrochloride (L-NAME), D-NAME (isomer of L-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), spermine NONOate, trequinsin and thrombin. BAPTA-AM and ODQ were dissolved in dimethyl sulfoxide (DMSO) and diluted to a final DMSO concentration of 0.06%. Perkin-Elmer Life and Analytical Sciences, Inc. (Boston, MA) supplied the [carboxyl-14C]inulin (molecular weight range 5000-5500, 2 mCi/g).

Cell Culture – HUVEC were obtained from ScienCell Research Laboratories (Carlsbad, CA) and cultured in endothelial cell medium with included supplements from the same company. They were used between passages 3-10. EA.hy926 cells (provided by Dr. Cora Edgell, University of North Carolina, Chapel Hill, NC) were cultured in Dulbecco’s minimal essential medium containing 10% (v/v) heat-inactivated fetal bovine serum, 20 mM D-glucose and HAT media supplement (Sigma). EA.hy926 is an immortalized hybridoma line derived from HUVEC. They retain features of endothelial cells in culture, including a cobblestone appearance with formation of capillary-like tubes (12) and calcium-dependent endothelial nitric oxide synthase activity (13,14). Cells were cultured at 37 ºC in humidified air containing 5% CO2.

Assay of trans-endothelial inulin transfer – Cells were cultured 4-5 days past confluence in 6-well plates on polyethylene terephthalate cell culture inserts (0.4 micron pores at a density of 2 + 0.2 x 10⁶ pores per cm², Falcon BD Biosciences, Franklin Lakes, NJ) with 1.7 ml of medium in the upper well and 2.8 ml of medium in the lower well. After treatment with agents as indicated, 0.1 µCi of [carboxyl-14C]inulin was added to the upper well and cells were incubated at 37 ºC. After 60 min, medium above and below the cells/filter was sampled for liquid scintillation counting of radiolabeled inulin. The permeability of the endothelial cell layer to [carboxyl-14C]inulin was calculated as previously described (15), with minor modification (6). The calculated permeability coefficients for [carboxyl-14C]inulin were corrected for the rate of [carboxyl-14C]inulin transfer across filters after removal of cells (16). This accounted for any changes in permeability due to deposition of the matrix laid down by the cells during culture.

Assay of ascorbate and GSH – To measure intracellular ascorbate, cells cultured in 6-well plates were rinsed 3 times with Krebs-Ringer Hepes buffer (KRH) containing 20 mM Hepes,
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128 mM NaCl, 5.2 mM KCl, 1 mM NaH₂PO₄, 1.4 mM MgSO₄, and 1.4 mM CaCl₂ at pH 7.4. Following removal of the last rinse, 0.1 ml of 25 % (w/v) metaphosphoric acid was added to the cell monolayer, the cells were scraped with a rubber spatula, and the acid was partially neutralized with 0.35 ml of 0.1M Na₂HPO₄ and 0.05 mM EDTA, pH 8.0. The cell lysate was centrifuged at 4 °C for 1 min at 13,000 x g, and the supernatant was taken for assay of ascorbate in duplicate by high performance liquid chromatography as previously described (17). GSH was measured in the same sample as ascorbate by the method of Hissin and Hilf (18). Intracellular ascorbate and GSH concentrations were calculated based on the intracellular distribution space of 3-O-methylglucose in EA.hy926 cells relative to the cell protein content, which was previously measured to be 3.6 + 1.2 µl/mg protein (14).

**F-actin quantification** – Total F-actin content of cells was measured as described previously (19). Briefly, cells grown to confluence in 12-well plates were fixed and stained with rhodamine phalloidin as described above. Cells were washed three times with PBS and the fluorescent dye was eluted by incubation with methanol for 40 min. Fluorescence intensities of eluents were quantified in duplicate with a fluorescence microtiter plate reader (Synergy HT multi-mode microtiter reader; Biotek, Winooski, VT). Complete de-staining of cells was confirmed using fluorescence microscopy.

**Western blot analysis** – Near-confluent cells were lysed with RIPA Buffer (catalog # R0278, Sigma), and immunoblotting was performed as described previously (20). Briefly, protein yield was quantified using a BCA assay (catalog # 23225, Pierce Biotechnology, Rockford, IL). Normalized samples were prepared with Laemmli sample buffer containing 5% β-mercaptoethanol and electrophoresed on a 4-20% SDS-polyacrylamide gel. Proteins were transferred to polyvinylidene difluoride membranes and probed with goat anti-VE-cadherin (catalog # 6458, Santa Cruz Biotechnology, Santa Cruz, CA; 1:5000), rabbit anti-phosphorylated Tyr-731 of VE-cadherin (pVE-cadherin; catalog # ab27776, Abcam, Cambridge, MA; 1:20000), mouse anti-myosin light chain (MLC; catalog # M4401, Sigma; 1:10000) and rabbit anti-phosphorylated Ser-19 of MLC (pMLC; catalog # ab2480, Abcam; 1:1000). Antibody binding was detected with enhanced chemiluminescence reagent (catalog # NEL105001EA, Perkin Elmer) using 1:5000 horseradish peroxidase-conjugated secondary antibodies (catalog # W4011 and W4021, Promega Corporation, Madison, WI; catalog # A5420, Sigma). Signal intensity was analyzed within a linear range using ImageJ (National Institutes of Health, Bethesda, MD).

**Immunoprecipitation** – Active Rac1, Rap1 and RhoA were immunoprecipitated using kits from Cell Signaling (catalog #s 8815 and 8818) and Cytoskeleton, Inc. (catalog # BK036-S), following the manufacturers’ protocols. Briefly, total-protein-normalized lysates were incubated with a GSH resin and GST-bound PAK1-PBD, RaIGDS-RBD or Rhotekin-RBD at 4 °C for 1 h. The resin was then washed and incubated with 2x Laemmli sample buffer to elute the GTP-bound proteins. Eluents were electrophoresed and transferred as described above, then probed with mouse anti-Rac1 (1:2,000), rabbit anti-Rap1 (1:500) or mouse anti-RhoA (1:500). Total Rac1, Rap1 and RhoA in the starting samples were also measured by Western blotting to assess equal protein loading.

**Immunofluorescence staining and microscopy** – HUVEC were grown on glass coverslips coated with poly-L-lysine and fixed with 4% formaldehyde for 15 min. Cells were blocked with 10% donkey serum (Sigma), permeabilized with 0.1% saponin and probed for VE-cadherin (Santa Cruz; 1:200). Following incubation with an Alexa Fluor 488-conjugated secondary antibody (catalog # A11055, Life Technologies, Carlsbad, CA; 1:500), filamentous actin (F-actin) was stained with rhodamine phalloidin (Life Technologies; 1:100) and nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Cells were visualized using an Olympus FV1000 inverted confocal microscope with a 40x/1.3 Plan-NeoFluor oil-immersion lens at room temperature with FV10-ASW 1.7 acquisition software (Olympus Corporation, Tokyo, Japan).

**Measurement of intracellular calcium** – Intracellular calcium was measured using the
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Fluo-4 NW Assay Kit (Cat. # F36206, Molecular Probes) following the manufacturer’s protocol. Briefly, cells were cultured to confluence in 96-well plates and treated with either 0 or 200 µM DHA and with Fluo-4 NW reagent. After 1 h, cells were treated with either 0 or 1 U/mL of thrombin and fluorescence intensity was quantified with a fluorescence microtiter plate reader every 30 seconds for 20 min (Synergy HT).

cAMP and cGMP assays – cAMP and cGMP content were measured using competitive enzyme-immunoassay systems according to the manufacturer’s protocols (Cayman Chemical Company, Ann Arbor, MI). Briefly, cells cultured to 90% confluence were first treated with or without 200 µM DHA for 30 min. Cells were then treated with combinations of 2.5 mM L-NAME or D-NAME for 30 min, 10 µM ODQ for 30 min, 50 µM trequinsin for 15 min, 1 mM spermine NONOate for 15 min and/or 10 U/ml thrombin for 5 min. Cells were washed with PBS and lysed with RIPA Buffer. A 96-well plate coated with mouse anti-rabbit IgG was incubated overnight at 4 °C with rabbit-anti-cAMP/GMP antisera, cAMP- or cGMP-bound acetylcholinesterase solution and either cell lysates or known cAMP standards. The plate was then washed and incubated for 2 h with 5,5′-dithiobis-(2-nitrobenzoic acid), a color-changing substrate for the competitively bound cAMP/GMP-acetylcholinesterases. Colorimetric measures were made with a microtiter plate reader (Synergy HT) and converted to cAMP/GMP quantities using an Excel sheet included by Cayman. Protein quantities of lysates were subsequently measured with a BCA assay.

Data Analysis – Results are shown as mean ± standard deviation or standard error, as noted. One-way analysis of variance was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA), followed by Dunnett’s or Tukey’s post-hoc tests, as appropriate. Differences with P<0.05 were considered significant.

RESULTS
Intracellular ascorbate blocks thrombin-induced increases in endothelial permeability – The tightness of the endothelial barrier generated by EA.hy926 cells when cultured on semi-porous filters was assessed as the transfer of radiolabeled inulin across this barrier from the apical (luminal) to basolateral side of the cell layer. Increasing concentrations of thrombin added to the luminal chamber for 60 min increased transfer of radiolabeled inulin across the endothelial barrier, reaching statistical significance at a thrombin concentration of 0.15 U/ml (Fig. 1A). Since transfer rates plateaued above this level, 0.15 U/ml thrombin was used for subsequent permeability measures. Transfer rates were significantly increased compared to control at 45 min and were maximal at 60 min (results not shown), so 60 min was used for subsequent experiments.

EA.hy926 cells are exposed to very low concentrations of extracellular ascorbate in culture (about 4 µM (8)). To assess the effects of intracellular ascorbate on thrombin-induced permeability, we used DHA. DHA is the two-electron-oxidized form of ascorbate that is taken up on glucose transporters and is rapidly reduced to ascorbate inside the cells (21). Thus, loading cells with DHA rather than ascorbate itself avoids the potentially confounding effects of extracellular ascorbate such as redox cycling with ferric iron in the culture medium, which generates oxidative stress. After pre-treating cells with DHA for 60 min, the thrombin-induced increase in endothelial permeability was blunted in a dose-dependent manner by increasing concentrations of intracellular ascorbate (Fig. 1B). Even when cells were exposed to thrombin for 30 min before addition of DHA at the beginning of the inulin transfer assay, the expected thrombin-induced increase was still reversed at even the lowest DHA concentration (Fig. 1C). Thus, loading the cells with ascorbate either before or during thrombin treatment prevented the thrombin-induced increase in endothelial permeability.

Since EA.hy926 is an immortalized line and may no longer behave in the same manner as its primary cell equivalent, the inulin transfer experiment was repeated with HUVEC, adding DHA and thrombin simultaneously for 30 min before the radiolabeled inulin assay. As shown in Fig. 1D, thrombin increased HUVEC permeability to inulin, and this was again progressively decreased by ascorbate loading of the cells with DHA in a manner similar to that observed with EA.hy926 cells.

Compared with other antioxidants, ascorbate...
Ascorbate prevents endothelial permeabilization by thrombin and is consumed in the process – To determine whether thrombin generated intracellular oxidative stress, its effects on intracellular ascorbate and GSH were measured. EA.hy926 cells were treated for 15 min with 100 µM DHA to generate an intracellular ascorbate concentration of 1.3 mM (Fig. 2A). Subsequent treatment of the cells with increasing amounts of thrombin for 60 min caused a progressive decrease in intracellular ascorbate with statistical significance reached at a thrombin concentration of 0.1 U/ml. Thrombin did not significantly affect basal levels of the endogenous antioxidant GSH in cells, although there was a tendency for GSH to increase (Fig. 2B). Since thrombin did not increase the release of ascorbate into the medium (results not shown), these data suggest that thrombin caused a selective loss of ascorbate that was not seen with GSH.

If ascorbate lowers endothelial permeability and blocks thrombin-induced permeabilization through a general antioxidant effect, then other types of antioxidants should similarly stabilize the barrier. However, neither the cell-penetrant nitroxide Tempol, nor the low molecular weight dithiothreitol decreased basal endothelial permeability (Fig. 2C, bars three and five). Additionally, Tempol pre-treatment failed to block the increase in permeability by thrombin (Fig. 2C, bars three and four). Although dithiothreitol did significantly blunt this increase, the effect was only partial (Fig. 2C, bars five and six). While the loss of intracellular ascorbate suggests that thrombin generates oxidative stress in EA.hy926 cells, failure of other antioxidants to completely block thrombin-induced permeability indicates that ascorbate may do so, at least in part, via an alternate mechanism.

Ascorbate prevents thrombin-induced internalization of VE-cadherin and formation of actin stress fibers – To investigate the interplay of ascorbate and thrombin at a cellular level, two hallmarks of endothelial dysfunction were evaluated: F-actin remodeling and loss of VE-cadherin-mediated cell-cell adhesion. Fig. 3 shows confluent HUVEC monolayers stained for F-actin and VE-cadherin. Both untreated cells and cells treated for 30 min with 200 µM DHA (top two rows) displayed intact adherens junctions bordering cells in unbroken, confluent monolayers. Upon 10 min treatment with 2 U/ml of thrombin, VE-cadherin was found to localize less peripherally and more intracellularly. As a result, adherens junctions were diminished and gaps appeared between cells. However, pre-incubation for 30 min with DHA before thrombin exposure attenuated this effect, resulting in an appearance similar to untreated and DHA-treated cells.

F-actin in control and ascorbate conditions localized cortically. In contrast, exposure to thrombin diminished cortical actin and caused cytoskeletal remodeling to form actin stress fibers. In the merge panel for thrombin treatment, stress fibers are shown to bind and terminate at peripheral VE-cadherin, forming “discontinuous” adherens junctions (22). These junctions are related to dynamic remodeling of the endothelium and increased permeability across its barrier because the physical linkage of VE-cadherin to stress fibers allows tension along the fibers to pull endothelial cells apart (23). Pre-incubation with DHA before thrombin exposure did not completely prevent focal associations of F-actin and VE-cadherin. Even so, ascorbate markedly decreased the formation of stress fibers. As a result, VE-cadherin junctions could not be pulled apart, and cell-cell junctions were visibly maintained, promoting a tighter barrier.

Ascorbate prevents stress fiber formation by regulating F-actin polymerization and the activation of RhoA and MLC – Endothelial permeability occurs in part due to the sequential formation and contraction of stress fibers, comprised of F-actin and myosin II. To investigate the mechanism by which ascorbate prevented stress fiber formation, we first evaluated the polymerization of F-actin in HUVEC. Cells were stained with rhodamine phalloidin for F-actin, the fluorescent dye was eluted with methanol and relative F-actin quantities were compared between treatment conditions (Fig. 4A). Exposure to 2 U/ml thrombin for 15 min caused a 34% increase in F-actin relative to basal, while the F-actin content of cells pre-treated for 30 min with 200 µM DHA before thrombin exposure did not significantly change. Although not significant, treatment with DHA alone reduced F-actin to 7% below basal. These data indicate that ascorbate prevents
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thrombin-induced increases in actin polymerization and shows a tendency to decrease F-actin quantity in unstimulated cells.

The driving force of stress fiber formation is the phosphorylation of MLC, which promotes the interaction of F-actin with myosin. Because immunocytochemistry demonstrated a visible decrease in thrombin-induced stress fiber formation with the addition of intracellular ascorbate (Fig. 3), we next investigated the ascorbate effect on MLC phosphorylation. Western blot analysis was used to compare the amounts of total MLC to MLC phosphorylated at Ser-19 in HUVEC (Fig. 4B). While ascorbate alone did not significantly affect phosphorylation, a 15 min treatment with 2 U/ml thrombin increased phosphorylation to 88% above basal. Adding DHA before thrombin significantly prevented the thrombin-induced increase in phosphorylation of MLC.

RhoA, a small GTPase, not only increases phosphorylation of MLC by inhibiting MLC phosphatase, it also increases total F-actin by inhibiting cofilin-mediated actin depolymerization. To explain the role of ascorbate in both of these effects, cells were treated with or without 200 µM DHA and 2 U/ml thrombin. Western blot analysis was used to compare immunoprecipitated active RhoA-GTP against total RhoA from whole cell lysates. While untreated cells expressed very little active RhoA, activation was increased almost 21-fold following a 5 min exposure to thrombin. A 30 min pre-treatment with DHA alone did not affect basal RhoA activation, but it partially blunted thrombin-induced activation, taking active RhoA down to a 12-fold increase above basal.

Ascorbate stabilizes peripheral actin and cell-cell junctions by maintaining activity of Rap1 and Rac1 in addition to preventing VE-cadherin phosphorylation – We examined whether the small GTPases Rap1 and Rac1, which have been shown to maintain cortical actin, might be involved the ability of ascorbate to protect cortical actin from degradation (Fig. 3). Following pre-treatment with 200 µM DHA for 30 min and treatment with 2 U/ml thrombin for 30 min, Western blot analysis was used to compare immunoprecipitated active GTPases against total GTPases from whole cell lysates. Ascorbate did not significantly affect basal levels of either active Rap1 or Rap1 (Fig. 5A and 5B). Thrombin stimulation induced a 25% decrease in Rap1 activation, which was prevented by pretreatment with DHA. Thrombin also decreased Rac1 activation by 68%, which was partially prevented (to 29%) by DHA.

Tyr-731 phosphorylation of VE-cadherin is not well understood. However, tyrosine phosphorylation is a well-characterized cause of VE-cadherin junction opening and increased vascular permeability (24). Phosphorylation has been linked to both endocytosis of VE-cadherin (25) as well as the dissociation of VE-cadherin from β-catenin and subsequent loss of stable association with the cortical actin cytoskeleton (26). Because we observed ostensible internalization of VE-cadherin (Fig. 3), Western blot analysis was used to determine if and how phosphorylation status was being altered. Comparing pVE-cadherin to total VE-cadherin, thrombin increased phosphorylation of VE-cadherin by 67%, and this effect was completely prevented by pre-treatment with DHA (Fig. 5C). However, ascorbate alone did not significantly reduce phosphorylation below basal levels.

Role of intracellular calcium in the effects of thrombin and ascorbate on endothelial permeability – The roles of thrombin in activating the above-mentioned GTPases as well as in increasing phosphorylation of MLC and VE-cadherin have been linked to its ability to increase intracellular calcium (27). Thus, decreasing the availability of extracellular calcium and presumably intracellular calcium might limit barrier permeabilization by thrombin. This was tested by comparing inulin permeability in Ea.hy926 cells with or without calcium in the medium. Permeability with no calcium was set to a value of 1, and was not found to differ significantly from permeability with calcium in the medium (Fig. 6A, first bar). However, omission of medium calcium did prevent the thrombin-induced increase in inulin transfer (Fig. 6A, bars two and three). As expected (6), ascorbate loading alone significantly decreased basal rates of inulin transfer (Fig. 6A, bars one and four). Although omission of medium calcium tended to reverse the decrease in basal endothelial permeability due to ascorbate loading with DHA, this did not reach
statistical significance (Fig. 6A, bars four and five).

Another approach to decrease intracellular calcium is to treat the cells with the cell-penetrant calcium chelator BAPTA-AM. Once inside cells, the acetoxyethyl groups of BAPTA-AM are removed, generating BAPTA that can then chelate intracellular calcium. Results are shown in Fig. 6B as a fraction of untreated cells, since they are from several independent sets of experiments. As with removing calcium from the medium, treatment of EA.hy926 cells for 30 min with 3 µM BAPTA-AM did not affect basal inulin transfer (Fig. 6B, first bar). However, it completely reversed the increase in endothelial permeability due to thrombin (Fig. 6B, bars two and three). It also prevented the 40% decrease in basal endothelial permeability in response to ascorbate loading with DHA (Fig. 6B, bars four and five). Together, the results with removing medium calcium and BAPTA treatment suggest that normal amounts of intracellular calcium are necessary for the opposite changes in endothelial permeability due to both thrombin and ascorbate.

Next, the calcium ionophore A23187 was used to increase intracellular calcium by allowing its passage from the medium into the cells. In the absence of intracellular ascorbate, a 6 µM concentration of A23187 caused a 4-fold increase inulin permeability compared to basal rates (Fig. 6C, square and circle at zero DHA). As was seen with thrombin, loading the cells with increasing amounts of ascorbate as DHA progressively prevented the increase in endothelial permeability caused by A23187 (Fig. 6C, open circles). HUVEC had a two-fold increase in permeability due to 6 µM A23187 that was also prevented by ascorbate loading (results not shown). Although intracellular ascorbate prevented the increase inulin transfer, as with thrombin, it did not decrease transfer below basal levels.

However, the ability of ascorbate to block thrombin-induced increases in endothelial barrier permeability was not due to any effect of ascorbate on intracellular calcium availability, as shown in Fig. 6D. Changes in intracellular calcium were measured qualitatively as increased fluorescence of Fluo-4. Although thrombin caused a rapid increase in intracellular calcium over several minutes, ascorbate loading neither had an effect alone (Fig. 6D, circles) nor modified the increase due to thrombin (Fig. 6D, triangles). Together, these results suggest that although tightening of the endothelial barrier by ascorbate requires calcium, its impact on calcium-dependent increases in endothelial permeability either occurs downstream of the calcium effect or is independent of it.

Ascorbate attenuates cAMP reduction by thrombin in a NO and cGMP-dependent manner – In addition to stimulating calcium influx, thrombin has been shown to decrease availability of intracellular cAMP in endothelial cells (28), an effect that could explain its activation of small GTPases and phosphorylation of MLC and VE-cadherin. To explore the interaction of ascorbate and thrombin on cAMP levels, HUVEC were loaded with ascorbate using 200 µM DHA, treated with 10 U/ml thrombin for 5 min, lysed and subjected to a colorimetric ELISA for cAMP. Whereas thrombin decreased cAMP levels to 40% basal, pre-incubation with DHA partially attenuated this effect, resulting in a drop to 80% basal cAMP (Fig. 7A, bars one, three and four). Ascorbate alone did not significantly change cAMP levels.

We hypothesized that ascorbate, which recycles tetrahydrobiopterin (7) – a necessary cofactor for eNOS – took effect by up-regulating NO production. NO activates guanylate cyclase, and cGMP is a known inhibitor of PDE3, which eliminates cAMP. To determine if ascorbate worked through eNOS, cells were simultaneously incubated for 30 min with DHA and 2.5 mM L-NAME, an eNOS inhibitor, or D-NAME, the inactive stereoisomer of L-NAME. Cells were then exposed to thrombin. Inhibition of eNOS with L-NAME prevented ascorbate from blunting the thrombin effect, as cAMP levels were statistically the same as treatment with thrombin alone (Fig. 7A, bars three and five). Treatment with D-NAME, however, did not statistically change cAMP levels from combined DHA and thrombin treatment alone (Fig. 7A, bars four and six). Finally, cells were treated for 15 min with 1 mM spermine NONOate, a NO donor, followed by treatment with thrombin. Spermine NONOate attenuated the thrombin effect in a manner similar to that observed for ascorbate (Fig. 7A, bars four and seven).

To confirm the involvement of guanylate
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cyclase and cGMP, we performed permeability assays using ODQ, a highly selective guanylate cyclase inhibitor. 10 µM ODQ for 30 min did not affect basal permeability in HUVEC (Fig. 7B, bars one and three). While ascorbate normally brings thrombin-induced permeability back to basal, inhibiting guanylate cyclase with ODQ prevented this ascorbate effect, maintaining the increase in permeability with thrombin (Fig. 7B, bars four through six).

Because we hypothesize that ascorbate works by inhibiting PDE3, the selective PDE3 inhibitor trequinsin should mimic the ascorbate effect on permeability. 50 µM of trequinsin for 15 min decreased permeability below basal levels in HUVEC, similar to ascorbate (Fig. 7C, bars one through three). Likewise, both ascorbate and trequinsin independently prevented thrombin-induced permeability, preserving basal levels (Fig. 7C, bars four through six).

To further validate cGMP as a relevant downstream effector of ascorbate, we repeated the aforementioned treatments with DHA, ODQ, trequinsin and spermine NONOate, then treated HUVEC with 10 U/ml thrombin for 5 min and measured lysates for cGMP with a colorimetric ELISA. Ascorbate alone was found to increase cGMP more than 2-fold over basal levels, while thrombin did not significantly affect cGMP and did not diminish increases by ascorbate (Fig. 7D, bars one through four). These findings are consistent with our hypothesis that an increase in cGMP occurs downstream of ascorbate but upstream of thrombin signaling.

While inhibiting guanylate cyclase with ODQ alone diminished cGMP, it also prevented ascorbate from increasing cGMP in thrombin-stimulated conditions (Fig. 7D, bars four through six), corroborating our data from permeability measures with ODQ (Fig. 7C, bars five and six). Furthermore, inhibition of PDE3 with trequinsin did not change basal cGMP levels in both unstimulated and thrombin-stimulated cells (Fig. 7D, bars seven and eight), verifying that cGMP occurs upstream of the thrombin signaling cascade. Finally, increasing NO with spermine NONOate increased cGMP to more than 3-fold basal levels, confirming that increasing NO with ascorbate could sufficiently stimulate cGMP production. Together, the data from Fig. 7 suggest the ascorbate effects on guanylate cyclase, cGMP and PDE3 are sufficient to preserve cAMP and promote endothelial barrier integrity.

DISCUSSION

Thrombin is a serine-protease that cleaves specific residues on protease-activated receptors (PARs) in endothelial cells. Following thrombin stimulation, these G-protein-coupled receptors activate a dual signaling cascade that results in actin remodeling and weakening of the endothelial permeability barrier (Fig. 8). First, it activates RhoA in a calcium-dependent manner (2,29). RhoA is a small GTPase that, when bound to GTP, activates Rho kinase, which then phosphorylates and inactivates a myosin-associated phosphatase (30-32). Loss of this phosphatase activity results in increased phosphorylation of Ser-19 on the light chain of myosin II, which undergoes a conformational change that allows it to associate with actin and induce cytoskeletal rearrangement to form actin stress fibers (33). This results in cell contracture and opening of gaps between endothelial cells, through which serum proteins and other molecules can pass from the blood into the tissue.

The second arm of the thrombin signaling cascade causes down-regulation of cAMP (28,34). PAR receptor activation by thrombin both inhibits adenylate cyclase via G_i (35) and activates phosphodiesterase 3 (PDE3), a cGMP-inhibited cAMP phosphodiesterase (36,37). The resulting decrease in cAMP impairs function of signaling pathways involving both protein kinase A and Epac1. Epac1 is required for the sequential activation of Rap1 and Rac1 (38,39). When active, both of these small GTPases maintain cortical actin (40). Rap1 activates Rac1, and through this signaling axis these molecules induce translocation of cortactin to the cell borders, strengthening the cortical actin cytoskeleton (41). Additionally, Rac1 has been shown to act through LIM kinase to phosphorylate and inhibit cofilin, preventing cofilin-mediated depolymerization of cortical actin (42). Furthermore, activation of PKA by cAMP and activation of p115RhoGEF by Rac1 have both been shown to inhibit RhoA activity and prevent actin stress fiber formation (43,44). Thus, by decreasing available cAMP, thrombin inhibits both of these signaling arms, pushing the molecular balance further toward barrier...
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destabilization. This dual-armed signaling cascade is represented in Fig. 8.

It is clear from the foregoing that there are multiple diverse mechanisms involved in thrombin-induced increases in endothelial permeability. Even so, all of these effects appear to be at least partly mediated by the availability of cAMP. The present studies demonstrate that ascorbate attenuates thrombin-induced barrier destabilization at multiple points within this signaling cascade (inactivation of Rap1 and Rac1, activation of RhoA, phosphorylation and distribution of VE-cadherin, phosphorylation of MLC, and actin polymerization), suggesting that it affects a step early in the cascade. Indeed, ascorbate was found to partially stabilize cAMP levels in the presence of thrombin in an NO- and cGMP-dependent manner. In previous studies using different cells, ascorbate either decreased basal cAMP (45-47) or increased cAMP under stimulatory conditions (48,49). Our results clarify previous findings by showing that ascorbate preserves endothelial cAMP in the face of thrombin stimulation.

There is a clearly defined mechanism by which ascorbate can increase cAMP. The vitamin is required for production of NO by eNOS because it recycles the cofactor tetrahydrobiopterin (7,50). NO in turn activates guanylate cyclase, and the resulting cGMP inhibits thrombin-induced permeability by competitively inhibiting PDE3 and subsequently increasing intracellular cAMP (4,51). By increasing cGMP through NO, ascorbate can thus inhibit PDE3 and blunt thrombin-induced down-regulation of cAMP. Moreover, because calcium serves as a cofactor for cNOS (52), this would explain why ascorbate requires the availability of calcium to tighten the endothelial barrier.

Although ascorbate preserved cAMP, it did not increase cAMP above basal levels. We speculate that this is because basal activity of PDE3 may be very low in unstimulated cells, such that ascorbate inhibition of PDE3 does not significantly affect the cAMP present. However, when thrombin up-regulates PDE3 activity, higher levels of cGMP via ascorbate (53) competitively bind and partially inactivate PDE3, diminishing the effect of thrombin. Whereas ascorbate alone tightened the endothelial permeability barrier to inulin transfer in this and a previous study (6), this finding was not explained by our signaling data and further studies will be needed to clarify this mechanism.

In conclusion, our findings suggest a novel role for ascorbate, but not other antioxidants, to protect vascular endothelial integrity and cytoskeletal structure. In response to the inflammatory mediator thrombin, this effect appears to be due to the ability of ascorbate to maintain intracellular cAMP through the NO-cGMP pathway.

Several conditions and diseases are characterized by both depleted ascorbate and vascular leakage, including diabetes (54), metabolic syndrome (55), inflammatory neurodegenerative diseases (54) and sepsis (56,57). Ascorbate supplementation to replete normal plasma levels (40-60 µM (7)) is indicated in most diseases where ascorbate is deficient, but is rarely made a priority. Our data emphasizes the importance of ascorbate supplementation to combat vascular dysregulation and proposes a novel mechanism by which ascorbate prevents inflammatory permeabilization of the endothelium.

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Author contributions: WHP and JMM conceived and coordinated the study and wrote the paper. WHP performed the experiments in Figures 3, 4, 5, 6 and 7, and ZCQ performed the experiments in Figures 1, 2.
and 7. Results were analyzed by WHP and JMM. All authors reviewed the results and approved the final version of the manuscript.
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FOOTNOTES

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2The abbreviations used are: BAPTA-AM, 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester); cGMP, cyclic guanosine monophosphate; DAPI, 4',6-diamidino-2-phenylindole; DHA, dehydroascorbic acid; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; eNOS, endothelial nitric oxide synthase; F-actin, filamentous actin; Hapes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; HUVEC, human umbilical vein endothelial cells; KRH, Krebs-Ringer Hapes; L/D-NAME, N-ω-nitro-L/D-arginine methyl ester hydrochloride; MLC, myosin light chain; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PBS, phosphate-buffered saline; PDE3, phosphodiesterase 3; VE-cadherin, vascular endothelial cadherin.

FIGURE LEGENDS

FIGURE 1. Ascorbate prevents thrombin-induced increases in endothelial permeability to inulin. Cells were cultured on porous membrane filters and culture medium was not removed prior to addition of agents for incubation and the transfer assay at 37 ºC. All additions were made to culture medium above the cells. A, Ea.hy926 cells were treated with increasing concentrations of thrombin as indicated just prior to the 60 min transfer assay. B, increasing concentrations of dehydrosascorbate (DHA) were added to the Ea.hy296 cells for 60 min, followed by vehicle (media; square) or addition of 0.15 U/ml thrombin just before the transfer assay (circles). C, Ea.hy926 cells were treated with 0.15 U/ml of thrombin (circles) or received no additions (square). After 30 min, the indicated concentration of DHA was added just before initiation of the inulin transfer assay. D, HUVEC were treated simultaneously with the indicated concentration of DHA and 0.15 U/ml of thrombin for 30 min prior to the radiolabeled inulin transfer assay. Results are from 4-6 experiments, and the values are expressed as means ± S.E. *, p < 0.05 versus sample not receiving thrombin (A) or versus all other samples (B-D).

FIGURE 2. Antioxidants and thrombin-induced endothelial permeability. A-B, Ea.hy926 cells cultured in 6-well plates to confluence were treated for 60 min in culture medium with the indicated concentration of thrombin followed by cell lysis and assay of intracellular ascorbate and GSH as described in Methods. C, EA.hy926 cells in culture on semi-porous filters were treated for 30 min without or with 0.3 mM concentrations of Tempol or dithiothreitol (DTT), followed by addition of 0.15 U/ml thrombin where indicated, and the inulin transfer assay. Results are from 4-6 experiments, and the values are expressed as means ± S.E. *, p < 0.05 versus the sample not treated with thrombin (A-B), or bars not having the same lower-case letters are different at p < 0.05 (C).

FIGURE 3. Presence and distribution of VE-cadherin and F-actin with ascorbate and thrombin treatment. HUVEC monolayers on glass coverslips were first treated with vehicle or 200 µM DHA for 30 min, then with or without 2 U/ml thrombin for 10 min. Cells were fixed and probed with rhodamine phalloidin and rabbit anti-VE-cadherin, followed by Alexa Fluor 488-conjugated donkey anti-rabbit antiserum. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for nuclei, and images were captured at 400x using an Olympus FV1000 inverted confocal microscope. Experiments were repeated 3 times, and representative images were chosen.

FIGURE 4. Ascorbate prevents formation of F-actin stress fibers by thrombin. A, confluent HUVEC were first treated with vehicle or 200 µM DHA (Asc) for 30 min, then with or without 2 U/ml thrombin (Thr) for 15 min. Cells were fixed and probed with rhodamine phalloidin. The molecule was then eluted with methanol and fluorescence intensity was compared between eluents on a microtiter plate reader.
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(Synergy HT). B, HUVEC treated as described above were lysed and analyzed by Western blotting for total myosin light chain (MLC) and MLC phosphorylated at Ser-19 (pMLC). C, HUVEC were treated as described above, except thrombin was added for only 5 min. Lysates were immunoprecipitated for GTP-bound RhoA (active RhoA). Western blot was used to analyze immunoprecipitated lysates for active RhoA and total cell lysates for total RhoA. Results are 3-6 experiments, and the values are expressed as means ± S.E. (A) or S.D. (B-C). Bars not having the same lower-case letters are different at p < 0.05.

**FIGURE 5. Ascorbate prevents degradation of cortical actin and cell junctions by thrombin.** A-B, confluent HUVEC were treated with vehicle or 200 µM DHA (Asc) for 30 min, followed 2 U/ml thrombin (Thr) for 30 min as indicated. Lysates were immunoprecipitated for the GTP-bound versions of Rap1 and Rac1 (active). Western blotting was used to analyze immunoprecipitated lysates for active Rap1 and Rac1 as well as total cell lysates for total Rap1 and Rac1. C, HUVEC were treated as described above, lysed and analyzed by Western blot analysis for total VE-cadherin and VE-cadherin phosphorylated at Tyr-731 (pVE-cadherin). Results are from 3-6 experiments, with the values are expressed as means ± S.D. Bars not having the same lower-case letters are different at p < 0.05.

**FIGURE 6. Calcium dependence on changes in endothelial permeability by ascorbate and thrombin.** A, medium both above and below EA.hy926 cells cultured on porous filters was replaced with KRH containing or excluding standard calcium, as indicated. This was followed by addition of 250 µM DHA or 0.15 U/ml thrombin as indicated just before the 60 min inulin transfer assay. Means are normalized relative to untreated control. B, EA.hy926 cells cultured on semi-porous filters were treated without or with 3 µM BAPTA-AM for 30 min, then with or without 250 µM DHA and 0.15 U/ml as indicated just before the inulin transfer assay. Means are normalized relative to untreated control. C, EA.hy926 cells cultured on filters were first treated with multiple indicated concentrations DHA for 30 min, then for 30 min with vehicle (0.06% dimethyl sulfoxide; square) or 6 µM A23187 (circles), followed by the transfer assay. D, The time course of changes in intracellular calcium were measured as fluorescence of Fluo-4 in EA.hy926 cells following treatment with 250 µM DHA (circles), 0.15 U/ml thrombin (squares), or both (triangles), as indicated. Results are from 3-7 experiments, with values expressed as means ± S.E. (A-C). Bars not having the same lower-case letters are different at p < 0.05 (A-B), or * indicates p < 0.05 versus untreated control (C). For D, results represent the means of two experiments with identical results, and S.E. was not shown to maintain visual simplicity.

**FIGURE 7. Ascorbate prevents reduction in cAMP by thrombin.** A, HUVEC were first treated with or without 200 µM DHA for 30 min, then 2.5 mM L-NAME or D-NAME for 30 min, 1 mM spermine NONOate for 15 min or 10 U/ml thrombin for 5 min, as indicated. Cells were lysed and cAMP content was determined by a colorimetric competitive ELISA using a microtiter plate reader (Synergy HT). cAMP is expressed relative to protein content, measured via BCA assay. B-C, HUVEC in culture on semi-porous filters were treated for 60 min with DHA, 30 min with 10 µM ODQ or 15 min with 50 µM trequinsin, then for and additional 30 min with 1.0 U/ml thrombin, as indicated. The inulin transfer assay was then performed as described in Methods. D, HUVEC were first treated with or without 200 µM DHA for 30 min, then 10µM ODQ for 30 min, 50 µM trequinsin for 15 min or 10 U/ml thrombin for 5 min, as indicated. cGMP was measured and is expressed in the same manner as cAMP. Results are from 3-6 experiments, and the values are expressed as means ± S.E. Bars not having the same lower-case letters are different at p < 0.05.

**FIGURE 8. Signaling mechanism by which intracellular ascorbate prevents endothelial permeabilization by thrombin.** * denotes molecules assayed in this paper.
Ascorbate prevents endothelial permeabilization by thrombin

Figure 1
Ascorbate prevents endothelial permeabilization by thrombin

Figure 2

Ascorbate (mM) vs. Thrombin (Units/ml)

GSH (mM) vs. Thrombin (Units/ml)

Permeability (cm/hr) vs. Thrombin, Tempol, DTT
Ascorbate prevents endothelial permeabilization by thrombin

Figure 3
Ascorbate prevents endothelial permeabilization by thrombin
Ascorbate prevents endothelial permeabilization by thrombin

Figure 5

![Bar charts and Western blots showing changes in active Rac1 and total Rac1 levels under different conditions.](image)

- **Panel A**: Shows active Rac1 levels with bars labeled 'Control', 'Ascorbate', 'Thrombin', and 'Asc + Thr'. Bars marked with lowercase letters 'a' differ significantly from 'Control'.
- **Panel B**: Similar to A, but with different comparison groups and letter markings.
- **Panel C**: Shows pVE-cadherin levels, with comparable trends and letter markings.

Note: The figure illustrates the role of ascorbate in modulating endothelial permeability in response to thrombin.
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Figure 6

[Image: Diagram showing the effects of calcium, thrombin, and ascorbate on permeability and intracellular calcium levels.]

A: Bar graph showing permeability (Fraction of Control) with and without calcium and thrombin in the presence of DHA.

B: Bar graph showing permeability (Fraction of Control) with and without BAPTA and thrombin in the presence of DHA.

C: Plot showing permeability (cm/h) with and without A23187 and dehydroascorbate (μM).

D: Plot showing intracellular calcium intensity with thrombin, ascorbate, and a combination of ascorbate and thrombin.
Ascorbate prevents endothelial permeabilization by thrombin

Figure 7

Graphs showing the effects of ascorbate on endothelial permeability and cAMP/protein levels in response to different treatments.
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Figure 8
Intracellular Ascorbate Prevents Endothelial Barrier Permeabilization by Thrombin
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