Ascorbate protects against vascular leakage in cecal ligation and puncture-induced septic peritonitis

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ABSTRACT

Vascular leakage in multiple organs is a characteristic pathological change in sepsis. Our recent study revealed that ascorbate protects endothelial barrier function in microvascular endothelial cell monolayers through inhibiting serine/threonine protein phosphatase 2A (PP2A) activation. The present study addressed the mechanism of protection by ascorbate against vascular leakage in cecal ligation and puncture (CLP)-induced septic peritonitis in mice. CLP caused NADPH oxidase activation and eNOS uncoupling to produce superoxide, increased NO production by iNOS and nNOS activity, and elevated 3-nitrotyrosine (a product of peroxynitrite) formation and PP2A activity in the hindlimb skeletal muscles at 12 h after CLP. The increase in PP2A activity was associated with decreased levels of phosphorylated serine and threonine in occludin, which was immunoprecipitated from freshly harvested endothelial cells of the septic skeletal muscles. Moreover, CLP increased the vascular permeability to fluorescent dextran and Evans blue dye in skeletal muscles. An intravenous bolus injection of ascorbate (200 mg/kg body weight), given 30 min prior to CLP, prevented eNOS-uncoupling, attenuated the increases in iNOS and nNOS activity, decreased 3-nitrotyrosine formation and PP2A activity, preserved the phosphorylation state of occludin, and completely inhibited the vascular leakage of dextran and Evans blue. A delayed ascorbate injection, given 3 h after CLP, also prevented the vascular permeability increase. We conclude that ascorbate injection protects against vascular leakage in sepsis by sequentially inhibiting excessive production of NO and superoxide, formation of peroxynitrite, PP2A activation, and occludin dephosphorylation. Our study provides a scientific basis for injection of ascorbate as an adjunct treatment for vascular leakage in sepsis.
INTRODUCTION

Vascular leakage is a critical pathological process in sepsis (29). It permits plasma protein and leukocyte extravasation, leading to edema and inflammatory reactions in the inflamed tissues (29). Edema causes tissue hypoxia. Leukocytes such as neutrophils cause tissue damage through the excessive production of free radicals and proteases. Vascular leakage is thus a promising target for therapeutic treatment. Sepsis is caused by a systemic response to infection, which is characterized by elevated levels of proinflammatory cytokines such as TNFα, IL-1β, IFNγ and IL-6 in the circulation or in the inflamed tissues (40). While the relevance of proinflammatory cytokines to vascular leakage has long been established (40), the molecular mechanism causing vascular leakage is not fully understood (29). Moreover, no specific therapy is available to treat this pathology (29).

*In vitro* studies of barrier function in endothelial and epithelial cell cultures have shown that proinflammatory stimuli decrease phosphorylation of serine and threonine residues in the tight junction protein occludin, stimulate redistribution of occludin from the cell borders to the cytoplasm, and increase paracellular permeability to macromolecules (22, 36, 43). It has also been observed, in endothelial and epithelial cell cultures, that serine/threonine protein phosphatase 2A (PP2A) dephosphorylates serine and threonine residues in occludin and this change is associated with disassembly of tight junctions and increased paracellular permeability (22, 36). Our recent studies of microvascular endothelial cells revealed that proinflammatory stimulus (LPS+IFNγ) increases the production of NADPH oxidase-derived superoxide and inducible nitric oxide synthase (iNOS)-derived NO, which form peroxynitrite to nitrate tyrosine
residues in the PP2A catalytic subunit (PP2Ac). This nitration in PP2Ac is associated with increase in PP2A activity, which then causes endothelial barrier dysfunction (37, 54).

The data from our recent studies of microvascular endothelial cell cultures indicated that intracellular ascorbate protects endothelial barrier function during septic insult and that this protection is associated with inhibition of oxidant production, PP2A activation, and occludin dephosphorylation and redistribution (22, 54). Pretreatment with ascorbate also attenuates the increase in monolayer permeability caused by LPS in aortic endothelial cell cultures (10). Furthermore, intravenous injection of ascorbate decreases edema formation in LPS-injected or burn-injured animals, as well as in patients with severe burn injury (11, 12, 15, 42, 48). The present study was designed to explore the mechanism of action of ascorbate in sepsis-induced vascular leakage. We tested the hypothesis that ascorbate injection prevents vascular leakage during sepsis by inhibiting the production of peroxynitrite precursors (NO and superoxide), the formation of peroxynitrite, and the consequent PP2A activation.
MATERIALS AND METHODS

Chemicals and reagents. Krebs solution and phosphate-buffered saline (PBS) were obtained from Invitrogen (Carlsbad, CA). p-Nitrophenyl phosphate (p-NPP) was from Calbiochem (Gibbstown, NJ); 1400W and N⁶-Propyl-L-arginine (NPA) were from VWR (West Chester, PA); anti-occludin antibody, anti-phosphothreonine antibody and anti-phosphoserine antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA); anti-PP2Ac antibody and protein A-agarose beads were from BD Biotechnology (Franklin Lakes, NJ); anti-3-nitotyrosine antibody and anti-tubulin antibody were from Millipore (Billerica, MA); and all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

Animals and sepsis model. Experiments were performed on male C57BL/6 mice (25–30 g) obtained from the Jackson Laboratory. The mice were provided with normal mouse chow and tap water ad libitum. The animal procedures were approved by IACUC at the University at Buffalo and complied with the American Physiological Society's "Guiding Principles in the Care and Use of Vertebrate Animals in Research and Training." Sepsis was induced by CLP, using the procedures described by us previously (51). Mice were anesthetized by ketamine (100 mg/kg body wt) plus xylazine (10 mg/kg body wt) given intraperitoneally. After laparotomy, the cecum was ligated distal to the ileocecal valve (bowel continuity preserved), punctured with an 18-gauge needle, and a small amount of cecal content was expressed through the puncture. Some mice were injected with a 0.1-ml bolus of sodium ascorbate (200 mg/kg body wt; dissolved in sterile 0.9% saline) through the tail vein at either 30 min before or 3 h after the CLP procedure. This dose of ascorbate (200 mg/kg body wt.) has been shown to block vascular leakage and improve survival in mice after CLP (52) or LPS injection (15). CLP mice were resuscitated
subcutaneously with a bolus of saline (50 ml/kg) containing buprenorphine (analgesic; 3 µg/ml). Control mice were subjected only to subcutaneous bolus injection of saline containing buprenorphine, because sepsis was defined in the present study as the outcome of the laparotomy and CLP procedures.

Although many organs become injured during sepsis, we studied skeletal muscle tissue. The rationale for choosing this tissue includes several considerations. First, during sepsis, edema is developed in subcutaneous tissue and body cavities in septic patients (29), suggesting widespread increases in vascular permeability. Secondly, CLP causes microvascular hyperpermeability and edema in the diaphragm and cremaster skeletal muscles (16, 25), suggesting that the microvessels in skeletal muscles are injured by CLP-induced systemic inflammation. Thirdly, cremaster skeletal muscles are accessible for intravital fluorescent microscopy. Vascular leakage in critical organ, such as the heart, lung, or diaphragm, dictates the outcome of sepsis. However, these organs are difficult to study with intravital microscopy, mainly because of their continuous movements. Therefore we choose skeletal muscles that are accessible to intravital microscopy and permit addressing the mechanism of vascular leakage. The underlying assumption is that the mechanism of vascular leakage in this tissue is comparable to that in other tissues.

_Tissue homogenate preparation._ Mice were sacrificed with cervical dislocation after anesthesia with ketamine plus xylazine at 12 h after CLP. The hindlimb skeletal muscles were collected using standard dissection methods and cleaned of excess fat, connective tissue, and tendons. The tissues were homogenized in 5 vol (1 mg wet wt/5 µl vol) of homogenization buffer (pH 7.4)
composed of 5 mM potassium phosphate, 250 mM sucrose, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol. The homogenates were aliquoted, snap-frozen in liquid nitrogen, and stored at –80°C for protein detection by Western blot analysis and for measurement of enzymatic activities.

Measurement of tissue superoxide production capacity. Tissue superoxide production capacity was measured by superoxide dismutase (SOD) + catalase-inhibitable oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCF diacetate). For each sample, 200 μg of tissue homogenate was added into a well of a 96-well white scintillation microplate and was mixed with a buffer containing 100 μM NADPH, 10 μM H₂DCF diacetate, and either one of 250 μM apocynin, 10 μM rotenone, 100 μM allopurinol, 1 mM N⁶-nitro-L-arginine methyl ester (L-NAME), or vehicle (DMSO). The mixture was incubated at 37°C for 30 min with or without manganese SOD (500 U/ml) + catalase (1000 U/ml). The fluorescence derived from H₂DCF diacetate oxidation was measured with a fluorescence spectrophotometer at excitation and emission wavelengths of 485 and 528 nm, respectively. Duplicate measures were performed for each sample. The superoxide production capacity was expressed as the differences in fluorescence between comparable wells with and without SOD + catalase.

Measurement of NOS activity. NOS enzymatic activity was measured by NOS inhibitor-sensitive fluorescence production by 4-amino-5-methylamino-2′,7′-difuorofluorescein (DAF-FM) as described previously (3). For each sample, 200 μg of tissue homogenate was put into a well of a 96-well white scintillation microplate and was mixed with vehicle (PBS), 100 μM 1400W, 10 μM NPA, or 1 mM L-NAME. This mixture was then incubated with PBS buffer containing 200
μM L-arginine, 2 mM calcium and 1 μM DAF-2 DA at 37°C for 1 h. The fluorescence was measured with a fluorescence spectrophotometer at excitation and emission wavelengths of 485 and 528 nm, respectively. Duplicate measures were performed for each sample. Total NOS activity was determined by calculating the difference in fluorescence between L-arginine and L-NAME. iNOS activity was the difference between L-arginine and 1400W. nNOS activity was the difference between L-arginine and NPA. eNOS activity was the difference between total NOS and iNOS + nNOS.

Western blot analysis. 50 μg of tissue homogenate protein was separated by 12% SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membrane. The blots were incubated first with primary antibodies and then with respective horseradish peroxidase-conjugated secondary antibodies, by standard procedures as we described previously (54). Protein bands were detected by ECL detection system and autoradiography film, and quantified with Quantity One software (Bio-Rad). Because of their inhibition of 3-nitrotyrosine detection, dithiothreitol and 2-mercaptoethanol were omitted from the homogenization buffer and the sample loading buffer, respectively, and sample heating was not performed in Western blot analysis of 3-nitrotyrosine (44). The blot was further probed with anti-tubulin antibody to normalize for protein loading. The intensity of each protein band was normalized to the respective tubulin band and then expressed as percentage of value for the control group.

Measurement of PP2A activity. PP2A activity was determined as okadaic acid-inhibitable phosphatase activity as we described previously (54). For each sample, 200 μg of tissue homogenate was mixed with 100 μl of assay buffer (5 mM p-NPP, 3 mM MnCl2, 0.1 mM
EDTA, 50 mM Tris-Cl, pH 7.0) with or without 5 nM okadaic acid. After incubation for 10 min at 30°C, the mixture was filtered through a 3 kDa molecular weight cutoff filter to eliminate proteins. The hydrolysis of p-NPP in the solutions was determined at 405 nm. PP2A activity was calculated as the difference between total phosphatase activity and okadaic acid-insensitive phosphatase activity.

*Intravital fluorescence microscopy.* At 12 h after CLP, the mice were anesthetized with ketamine plus xylazine. The jugular vein was cannulated for intravascular infusion of fluorescent dextran as described below. The cemaster muscle was dissected and exteriorized onto a histological glass slide with blood and nerve supplies preserved and suffused with physiologic Krebs solution (24). The preparation was following by a 30-minutes stabilization period. Then 50 mg/kg fluorescein isothiocyanate-labeled dextran 70 kDa was infused intravenously and fluorescence intensity in 2 or 3 areas of cremaster muscle was recorded by a digital camera. The fluorescence intensity in these areas was quantified with Quantity One software (Bio-Rad). The change of intensity, which reflected the fluorescent dextran leakage, was expressed as percentage of value for the control group.

*Endothelial cells isolated from skeletal muscle.* Microvascular endothelial cells were isolated from hind limb skeletal muscles using a cell-trapping technique as described by us (50). Briefly, muscle tissue was dissected out, minced into small pieces using dissecting scissors, and then digested in a collagenase solution. The tissue digests were filtered through a sterile 100 µm nylon mesh. The cells were pelleted by centrifugation, resuspended in DMEM medium, and incubated with magnetic beads coated with *Griffonia simplicifolia* lectin I. After incubation at
4°C for 20 min, the magnetic beads and bound cells were trapped with a magnet and washed four times with PBS containing 0.1% BSA. The beads and cells were then removed from the magnet and stored frozen at -80°C. These cells were used for immunoprecipitation of occludin and the latter was further analyzed by Western blot method for phosphothreonine, phosphoserine, and total occludin.

Immunoprecipitation. The frozen cells isolated from the muscles were lysed by sonication in 500 µl lysis buffer (20 mM Tris-HCl, pH 7.0, 1% NP-40, 137 mM NaCl, 1 mM PMSF, 1 µM pepstatin, and 1µM aprotinin). Nuclear and cellular debris were removed by centrifugation for 10 min at 12 000 rpm at 4°C. The supernatants were incubated with anti-occludin polyclonal antibody for 18 h at 4°C, followed by addition of protein A-agarose beads for 3 h at 4°C. The immunoprecipitates were washed four times with ice-cold lysis buffer and then were heated with sample loading buffer for electrophoresis and Western blot analysis of phosphothreonine, phosphoserine, and total occludin.

Statistics. Data are presented as mean ± standard error. They were analysed with the Prism statistical software (GraphPad Software, San Diego, CA). Comparisons between treatment groups were performed with one-way analysis of variance followed by the Tukey multiple comparison test. P<0.05 was considered significant.
RESULTS

Ascorbate attenuates CLP-induced production of superoxide, NO and peroxynitrite. We first determined the superoxide production capacity in the skeletal muscle of mice subjected to i.v. ascorbate injection and CLP procedure. The results showed that the tissue of control mice produced a high baseline level of superoxide that was sensitive to the NADPH oxidase inhibitor apocynin, but not to the mitochondria respiratory chain inhibitor rotenone, xanthine oxidase inhibitor allopurinol, or NOS inhibitor L-NAME, indicating that the high baseline level of superoxide was mainly synthesized by NADPH oxidase (Fig. 1). CLP significantly increased superoxide production and this effect was markedly inhibited by either apocynin or L-NAME (Fig. 1), indicating that both NADPH oxidase and NOS contribute to the elevated production. However, injection of ascorbate prior to CLP reduced the superoxide production to the baseline levels (Fig. 1). Notably, L-NAME did not further inhibit the superoxide levels (Fig. 1). Taken together, our results indicate that CLP increased superoxide production by NADPH oxidase and NOS, which was prevented by ascorbate.

Next we measured NOS activity. A baseline level of total NOS activity was detected in the skeletal muscle of control mice (Fig. 2A). CLP significantly increased the total NOS activity and this increase was abolished by ascorbate (Fig. 2A). We further investigated the effect of CLP on the activity of individual NOS isoforms. Baseline levels of iNOS and nNOS activities were detected in the skeletal muscle of control mice (Fig. 2B). CLP markedly increased the activities of iNOS and nNOS, which were inhibited by ascorbate injection (Fig. 2B). In contrast, a high level of eNOS activity was detected in the tissue of control mice, which was decreased by CLP (Fig. 2B). Ascorbate injection before CLP maintained eNOS activity at the level found in non-
septic control mice (Fig. 2B). These data show that the effects of CLP on NOS activities were prevented by ascorbate injection.

Superoxide reacts with NO to form peroxynitrite, which is a potent nitrating agent (47). We detected peroxynitrite product in tissue homogenates by Western blot analysis of 3-nitrotyrosine. Since it has been reported that denaturing conditions such as dithiothreitol, 2-mercaptoethanol, and sample boiling inhibit the detection of 3-nitrotyrosine (44), we omitted these conditions in our Western blot analysis. We detected proteins containing 3-nitrotyrosine at sizes of 55 kDa and 100 kDa in all samples. As shown in Fig. 3, very weak 3-nitrotyrosine bands were detected in the skeletal muscle tissue of control mice. However, CLP remarkably increased 3-nitrotyrosine-positive proteins and this effect was significantly attenuated by ascorbate. Taken together, the above data indicate that CLP upregulates the enzymatic activities that produce superoxide and NO. Furthermore, ascorbate inhibits the stimulation by CLP of these enzymatic activities and peroxynitrite formation.

Ascorbate inhibits CLP-induced increase in PP2A activity and preserves the phosphorylation state of endothelial occludin. The in vitro studies from our earlier experiments indicated that proinflammatory stimuli-induced PP2A activity dephosphorylates the phosphoserine and phosphothreonine residues in occludin, and that their dephosphorylation is associated with occludin redistribution and endothelial monolayer hyperpermeability (22, 54). Ascorbate pretreatment of the endothelial monolayers inhibited PP2A activity increase and consequently maintained the serine and threonine residues of occludin in phosphorylated state (22). Therefore,
the present study investigated the effects of ascorbate injection and CLP on the phosphorylation state of occludin in endothelial cells from isolated skeletal muscles.

We first determined the expression of PP2Ac in homogenates of hindlimb skeletal muscle. As shown in Fig. 4 A and B, CLP and ascorbate injection did not affect the expression of PP2Ac because the same levels of PP2Ac protein were detected in the control, CLP and ascorbate injection plus CLP groups. However, CLP increased PP2A phosphatase activity by 3 fold and ascorbate prevented this change (Fig. 4C). Moreover, CLP decreased the abundance of phosphorylated serine and threonine residues in occludin immunoprecipitates prepared from freshly isolated endothelial cells (Fig. 5). Prior injection of ascorbate in CLP mice maintained the phosphorylation state of endothelial occludin at the level found in non-septic control mice (Fig. 5). Taken together, these results suggest that ascorbate inhibits PP2A activation and preserves occludin phosphorylation state.

Ascorbate prevents CLP-induced vascular leakage. As mentioned above, our in vitro study demonstrated that ascorbate prevents proinflammatory stimuli-induced endothelial monolayer leakage by inhibiting PP2A-mediated dephosphorylation of occludin (22). The present experiment investigated if ascorbate prevents CLP-induced vascular leakage. After injection of fluorescent dextran, blood vessels in the cremaster muscle of non-septic control mice showed clear border lines that distinguished them from the surrounding tissue, indicating that no leakage occurred (Fig. 6A). CLP, however, caused fluorescent dextran leakage in many vessels, which made their borders appear more diffuse (Fig. 6A). Intensity analysis of the recorded areas indicated that CLP significantly increased the fluorescence intensity compared with control (Fig.
Nevertheless, ascorbate injection of mice prior to CLP maintained integrity of the vessels and decreased the fluorescence intensity to the level of the non-septic control (Fig. 5A and B). Similarly, CLP increased extravasation of Evans blue dye in hindlimb skeletal muscle (Fig. 7A). Injection of ascorbate before CLP completely blocked this leakage of the dye (Fig. 7A). To explore the therapeutic potential of ascorbate administered after the onset of sepsis, we delayed ascorbate injection until 3 h after CLP. This delayed ascorbate injection also completely prevented the stimulation by CLP of vascular leakage (Fig. 7B). Together, these results suggested that ascorbate prevents CLP-induced extravasation of macromolecules by maintaining the endothelial barrier.
DISCUSSION

Sepsis is associated with oxidative and nitrative stress (47). The present study indicates that peroxynitrite formation is associated with PP2A activity increase, occludin dephosphorytion, and vascular leakage. Importantly, prior ascorbate injection completely blocks the effects of CLP on these parameters, showing the mechanism by which ascorbate prevents vascular leakage in sepsis.

Our data indicate that CLP increased the production of peroxynitrite precursors, NO and superoxide, and the formation of peroxynitrite (as detected by 3-nitrotyrosine), which are associated with PP2A activation in skeletal muscle tissue. Indeed, it has been extensively reported that sepsis induces peroxynitrite formation. For example, increased levels of 3-nitrotyrosine have been detected in skeletal muscle, lung, liver, kidney, and gut in animal models of sepsis (1, 4, 9, 31, 38, 45). In humans, plasma 3-nitrotyrosine levels are undetectable in normal subjects but rise as high as 118 ± 22 μM in septic patients (17). During sepsis, LPS and proinflammatory cytokines, such as TNFα, IL-1β, and IFNγ, may stimulate NADPH oxidase activation and iNOS expression to generate superoxide and NO, respectively (39, 40). Indeed, in iNOS knockout and NADPH oxidase deficient mice, LPS induced much less production of NO and superoxide, respectively (7, 26). Moreover, CLP remarkably increases nNOS activity without affecting nNOS protein expression in mouse skeletal muscle (34). The results from the present study indicate that both NADPH oxidase and eNOS uncoupling are responsible for stimulation by CLP of superoxide production and that both iNOS and nNOS activity generate the excessive NO in septic skeletal muscles.
In vitro cell culture studies demonstrated that ascorbate not only scavenges superoxide but also inhibits iNOS induction and NADPH oxidase activity in various cell types exposed to inflammatory stimuli (14, 18, 19, 50, 52, 54). Further, ascorbate pretreatment of endothelial monolayers prevents PP2A activation by inhibiting iNOS induction and peroxynitrite formation (22, 54). Consistently, in the present study, we observed that injection of ascorbate in CLP mice prevents the increases in NO and superoxide production, the peroxynitrite formation and the PP2A activation in the skeletal muscle tissue. Specifically, ascorbate diminishes CLP-induced superoxide production largely through preventing NADPH oxidase activation and eNOS uncoupling. Further, ascorbate completely attenuates the CLP-induced increases in iNOS and nNOS activity, which otherwise synthesize excessive NO. The nNOS activity increase could occur through several mechanisms. Firstly, increased association with heat shock protein 90, which is up-regulated during sepsis (23), augments nNOS activity by enhancing the binding of calmodulin (46). Secondly, increased nNOS activity could be due to the decreased association with the endogenous NOS inhibitor, caveolin-3, which is downregulated in skeletal muscle during endotoxemia (35). However, the mechanism by which ascorbate inhibits nNOS activity in sepsis remains to be determined.

The endothelium forms a barrier that restricts the movement of blood cells and proteins across the vessel wall. Tight junctions formed between adjoining endothelial cells are the primary structures of the endothelial barrier (20). Serine and threonine phosphorylation is a major regulatory mechanism of the activity of tight junction proteins (13, 41). High levels of occludin phosphorylation are correlated with junction tightness (13, 41). Dephosphorylation of occludin, induced by cytokines, bacterial infection or peroxynitrite, is associated with disruption of tight
junctions and loss of barrier function in cell monolayers (6, 22, 32). The endothelial barrier
dysfunction induced by endotoxin and cytokines largely depends on PP2A activation, because
inhibition of PP2A activity by okadaic acid or siRNA knockdown of PP2Ac stabilizes the
endothelial barrier (54).

We observed that CLP increases PP2A activity in skeletal muscle. A consequence of PP2A
activity increase is dephosphorylation of the tight junction protein occludin, because the latter is
a substrate of PP2A (36). Studies on epithelial cells have demonstrated that occludin binds to and
is dephosphorylated by PP2A (36). Further, overexpression of PP2A catalytic subunit increases
the monolayer permeability to macromolecules such as insulin and mannitol, and this increase in
permeability is prevented by PP2A inhibitor okadaic acid (35). Similarly, our study of
microvascular endothelial monolayer cultures indicates that an increase in PP2A activity
dephosphorylates occludin, leading to occludin redistribution and endothelial monolayer leakage
(22). The present study further investigated if increase in PP2A activity in CLP-induced
peritonitis causes dephosphorylation of occludin. We isolated microvascular endothelial cells
from the septic skeletal muscles and immunoprecipitated occludin from the endothelial cell
lysates. Analysis of the occludin immunoprecipitates revealed that occludin is
dephosphorylated at the phosphoserine and phosphothreonine residues after CLP. These
results suggest that PP2A activity likely mediates the dephosphorylation of endothelial cell
occludin and causes vascular leakage in CLP mice.

Vascular leakage in multiple organs is a characteristic pathological change in sepsis (29). It
facilitates leukocyte and plasma protein extravasation, leading to inflammatory reactions and
edema. The latter ultimately causes tissue hypoxia and multiple organ dysfunction (29). Our study indicates that CLP disrupts vascular integrity through activation of PP2A. Nevertheless, other mechanisms could also be involved in this process of septic vascular injury. For example, vasoactive substances such as thrombin and histamine stimulate myosin light chain kinase-dependent contraction of the actin-myosin cytoskeleton, which leads to intercellular gap formation and transendothelial diffusion of macromolecules (28). Another example is the mechanism that mediates vascular endothelial growth factor (VEGF)-induced vascular leakage. VEGF activates the Src-Vav2-Rac1-PAK pathway, leading to phosphorylation and disassembly of VE-cadherin (21). The latter is an adherens junction protein that also contributes to endothelial barrier integrity (21). Thus, it is possible that multiple mechanisms act simultaneously to cause vascular leakage in sepsis.

Ascorbate is markedly depleted in septic patients and animals (5, 18, 30). Our previous studies indicated that prior bolus i.v. injection of ascorbate improves capillary blood flow, microvascular reactivity to vasoconstrictors, and survival in CLP mice (2, 51, 52). The current study discovered that ascorbate bolus i.v. injection, either prior to or after the onset of CLP, protects against the vascular leakage caused by CLP. Other studies have shown that i.v. administration of ascorbate decreases edema formation in LPS-injected or burn-injured animal models of critical illness (11, 12, 15, 42, 48). Furthermore, in a randomized, prospective, double-blind, placebo-controlled trial with 226 critically ill patients, 28-day mortality was decreased in the patients who received combined ascorbate and vitamin E by enteral feeding compared to those who did not (8). However, besides the beneficial effect described in the present study, ascorbate may act through other mechanisms to protect vascular integrity in sepsis. Since eNOS activity
has been shown to be barrier-protective in sepsis (33), ascorbate could act through preventing eNOS uncoupling and thus normalizing eNOS activity to maintain vascular integrity (49). Additionally, as CLP suppresses skeletal muscle translational efficiency (28), ascorbate may maintain protein synthesis to prevent vascular hyperpermeability.
Perspective and Significance

Edema develops in subcutaneous tissue and body cavities in septic patients because of widespread increases in vascular permeability (29). Edema causes tissue hypoxia that contributes to the development of multiple organ failure (29). Our earlier study found that endotoxin and cytokines induce iNOS protein expression in microvascular endothelial cells (50). Interestingly, the iNOS binds with and nitrates PP2A (37, 54). Nitration releases the A subunit from PP2A holoenzyme and increases the phosphatase activity (37). Increased PP2A activity causes dephosphorylation and redistribution of the tight junction-associated protein occludin from the plasma membrane to the cytoplasm, leading to barrier dysfunction (36). Ascorbate preserves the endothelial monolayer barrier integrity through inhibition of iNOS induction, PP2A activation, and occludin dephosphorylation (22, 50, 54). The present study revealed that ascorbate bolus injection similarly prevents vascular leakage in septic mice by inhibiting the production of peroxynitrite precursors (NO and superoxide), activation of PP2A, and dephosphorylation of occludin. Our study thus provides a scientific basis for parenteral administration of ascorbate as an adjunct treatment for vascular leakage in sepsis.
GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.
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FIGURE LEGENDS

**Fig. 1.** Ascorbate attenuates the increase in superoxide production in CLP mouse skeletal muscle tissue. Ascorbate (200 mg/kg) was injected i.v. in mice 30 min before CLP. At 12 h after CLP, hind limb skeletal muscles were harvested for assay. When assaying superoxide production, NADPH oxidase inhibitor apocynin (250 mM), mitochondrial respiratory chain inhibitor rotenone (10 μM), xanthine oxidase inhibitor allopurinol (100 μM), NOS inhibitor L-NAME (1 mM), or vehicle (DMSO) was added into the assay buffer. Data show the summary of superoxide production. n=5, *P<0.05 compared with Control; #P<0.05 compared with Vehicle within the group.

**Fig. 2.** Ascorbate attenuates NOS activity increase in CLP mouse skeletal muscle tissue. When assaying NOS activity, iNOS specific inhibitor 1400W (100 μM), nNOS specific inhibitor N-propyl-l-arginine (NPA, 10 μM), non-specific NOS inhibitor L-NAME (1 mM), or vehicle (PBS) was added into the assay buffer. **A:** Summary of total NOS activity. n=5, *P<0.05 compared with Control. #P<0.05 compared with CLP. **B:** Summary of NOS isoform activity. n=5, *P<0.05 compared with Control within the group; #P<0.05 compared with CLP within the group.

**Fig. 3.** Ascorbate inhibits 3-nitrotyrosine formation in CLP mouse skeletal muscle tissue. A. Representative Western blots of 3-nitrotyrosine and tubulin. B. Summary of 3-nitrotyrosine band intensity (expressed as percentages of the values for control group). n=6, *P<0.05 compared with Control; #P<0.05 compared with CLP.

**Fig. 4.** Ascorbate has no effect on PP2Ac protein expression but diminishes PP2A activity increase in CLP mouse skeletal muscle tissue. A. Representative Western blots of PP2Ac. B. Summary of PP2Ac band intensity expressed as percentage of the value for control group. C.
Summary of PP2A activity expressed as percentage of the value for control group. n=6, *P<0.05 compared with Control; #P<0.05 compared with CLP.

Fig. 5. Ascorbate preserves the phosphorylation state of occludin immunoprecipitated from microvascular endothelial cells of CLP mouse skeletal muscles. A. Representative Western blots of phosphothreonine, phosphoserine, and total occludin. B. Summary of phosphothreonine band intensity (expressed as percentage of the value for control group). C. Summary of phosphoserine band intensity (expressed as percentage of the value for control group). n=4, *P<0.05 compared with Control; #P<0.05 compared with CLP.

Fig. 6. Ascorbate prevents CLP-induced vascular permeability to fluorescent dextran in cremaster skeletal muscle. A. Representative recordings of fluorescent blood vessels. B. Summary of the vascular fluorescence intensity (expressed as percentages of the values for control group). n=5, *P<0.05 compared with Control; #P<0.05 compared with CLP.

Fig. 7. Effect of ascorbate, injected before or after CLP, on vascular leakage in mouse skeletal muscles. Ascorbate was injected i.v. either 30 min before or 3 h after CLP. A and B show the summaries of Evans blue extravasation (expressed as percentage of the value for Control). n=5, *P<0.05 compared with Control; #P<0.05 compared with CLP.
Figure 1

The bar chart shows the superoxide production (arbitrary units) in different treatments:

- **Control**
- **CLP**
- **Ascorbate + CLP**

Each treatment group is represented by different markers:

- **Vehicle**
- **Apocynin**
- **Rotenone**
- **Allopurinol**
- **L-NAME**

Significant differences are indicated by:

- *: Statistically significant difference compared to the control group.
- #: Statistically significant difference compared to the CLP group.

The chart illustrates the comparative effects of these treatments on superoxide production.
Figure 7

30 min before CLP

Evans blue leakage

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3 h after CLP

Evans blue leakage

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<td>100</td>
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</tbody>
</table>