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Ascorbate inhibits iNOS expression and preserves vasoconstrictor responsiveness in skeletal muscle of septic mice

Feng Wu,[1,2] John X. Wilson,[2] and Karel Tyml[1,2,3]

[1]*Lawson Health Research Institute, and Departments of* [2]*Physiology and Pharmacology and* [3]*Medical Biophysics, The University of Western Ontario, London, Ontario, Canada N6A 5C1*

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Wu, Feng, John X. Wilson, and Karel Tyml. Ascorbate inhibits iNOS expression and preserves vasoconstrictor responsiveness in skeletal muscle of septic mice. *Am J Physiol Regul Integr Comp Physiol* 285: R50–R56, 2003. First published March 13, 2003; 10.1152/ajpregu.00564.2002.—Inducible nitric oxide synthase (iNOS) expression in blood vessels contributes to the vascular hyporeactivity characteristic of sepsis. Our previous work demonstrated in vitro that ascorbate inhibits iNOS expression in lipopolysaccharide- and interferon-γ-stimulated skeletal muscle endothelial cells (ECs) through an antioxidant mechanism. The present study evaluated in vivo the hypothesis that administration of ascorbate decreases oxidative stress, prevents endothelial iNOS expression, and improves vascular reactivity in septic skeletal muscle. Sepsis was induced in C57BL/6 mice by cecal ligation and puncture (CLP). Plasma nitrite and nitrate (NOx) levels were elevated by 6 h after CLP. Prior ascorbate bolus injection (200 mg/kg body wt iv) blocked the elevation of plasma NOx and abolished the expression of iNOS protein and activity in the septic skeletal muscle. We also demonstrated that iNOS mRNA determined by RT-PCR was induced in the microvascular ECs of the muscle at 3 h after CLP. This induction was attenuated by prior ascorbate administration. Ascorbate inhibition of iNOS expression was associated with decreased oxidant levels in the septic muscle. Moreover, ascorbate administration restored partially the baseline arterial pressure and preserved completely the microvascular constriction and arterial pressure responses to norepinephrine in CLP mice. These results suggest that early administration of ascorbate may be a valuable adjunct treatment of sepsis.

oxidative stress; cecal ligation and puncture; arterial pressure; inducible nitric oxide synthase

IN SEPTIC PATIENTS AND ANIMALS, bacterial lipopolysaccharide (LPS) elicits the innate immune response and triggers the release of proinflammatory cytokines, including interleukin-1, interferon-γ (IFN-γ), and TNF-α. Induction of inducible nitric oxide synthase (iNOS) by LPS and proinflammatory cytokines contributes to the bactericidal effects of phagocytes (11, 16). LPS and cytokines also induce iNOS in vascular endothelial cells (ECs) and smooth muscle cells; this induction is, at least in part, responsible for vascular hyporeactivity, hypotension, and consequent multiple organ failure in sepsis (2, 33). In mice made septic by cecal ligation and puncture (CLP), vasoconstriction by norepinephrine (NE) in skeletal muscle arterioles is compromised (22). However, using the same model, arteriolar reactivity after CLP is maintained in iNOS-deficient mice. Moreover, these iNOS-deficient mice have improved survival (21). Although several drugs with relative selectivity for iNOS have been developed, their effectiveness in experimental sepsis therapy is limited because of their inhibition of all nitric oxide synthase (NOS) isoforms at relatively high doses (10, 25, 46).

Sepsis is also associated with elevated levels of reactive oxygen species (ROS; including superoxide, hydroxyl radicals, and hydrogen peroxide) (15, 27, 38, 47). ROS have been implicated in the potentiation of iNOS expression in a variety of cell types (3, 24, 44). Antioxidants (e.g., *N*-acetyl-L-cysteine, SOD/catalase) inhibit LPS- and cytokine-induced iNOS expression in ECs and other cell types (13, 32). Our recent in vitro results showed that ascorbate could blunt iNOS induction by LPS+IFN-γ in skeletal muscle ECs by reducing intracellular oxidative stress (45). However, endogenous ascorbate is depleted during sepsis (1, 4, 12). Although other antioxidants are also affected by sepsis, there is a strong correlation between survival and the plasma concentration of ascorbate (4, 15). Deficiency of endogenous ascorbate may worsen intracellular oxidative stress, thereby sensitizing cells to stimuli that induce iNOS. We hypothesize that administration of ascorbate can prevent sepsis induction of iNOS in vivo. The present study aimed to determine the effects of ascorbate administration on iNOS expression and vascular reactivity to NE in septic mice.

MATERIALS AND METHODS

Experiments were performed on male C57BL/6 mice (25–30 g) obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were provided with normal mouse chow and tap water ad libitum. The animal procedures were approved by the Council on Animal Care at the University of Western Ontario and complied with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings."

Address for reprint requests and other correspondence: K. Tyml, Dept. of Medical Biophysics, University of Western Ontario, London, ON, Canada N6C 5C1 (E-mail: ktyml@lhsc.on.ca).

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CLP. Sepsis was induced by CLP by using the procedures described by Armour et al. (1). Mice were anesthetized by 1% halothane (mixed with oxygen in the inspired gas). 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 30 min before the CLP procedure. The selected dose of ascorbate has been shown to prevent skeletal muscle microvascular dysfunction in CLP rats (1). CLP animals were resuscitated subcutaneously with a bolus of saline (50 ml/kg) containing buprenorphine (analgesic; 3 μg/ml). By 6 h after CLP, all of the mice had developed signs of severe sepsis, including lethargy, piloerection, and diarrhea. CLP caused 89% lethality at 24 h. 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.

Measurement of nitrate and nitrite. Plasma nitrite and nitrate (NOx) content was measured to estimate the total nitric oxide (NO) production. Samples were collected at the times indicated in the figure legends. Plasma was filtered through a 10-kDa molecular mass cutoff filter to eliminate proteins. Nitrate was converted to nitrite by nitrate reductase. Total nitrite was measured by using a total NO assay kit (Cayman, Ann Arbor, MI). Briefly, the reduced sample was mixed with an equal volume of Griess reagent, and the absorbance at 545 nm was measured immediately. The NOx concentration was determined by comparison to a standard curve on the basis of serial dilution of a sodium nitrate standard. The standard curve was linear over the range of 3–50 μM ($r = 0.998$).

Western blot analysis. Extensor digitorum longus (EDL) skeletal muscles were harvested from mice killed by cervical dislocation after anesthesia with ketamine (90 mg/kg) plus xylazine (4 mg/kg) given intraperitoneally at 6 h after CLP. The tissues from each animal were homogenized in 5 vol (wt/vol) of homogenization buffer (pH 7.4) composed of 5 mM potassium phosphate, 250 mM sucrose, 0.1 mM EDTA, 1 mM PMSF, and 1 mM dithiothreitol. For each sample, 200 μg of tissue homogenate protein were separated by 7.5% SDS-polyacrylamide gel electrophoresis and then transferred to a nitrocellulose membrane (Hybond ECL, Amersham Biosciences, Piscataway, NJ). For protein detection, blocked membranes were incubated with anti-NOS isoform antibodies (Transduction Laboratories, Lexington, KY) in phosphate-buffered saline containing 5% wt/vol nonfat dry milk and 0.01% Tween 20. Subsequently, the membranes were washed and treated with horseradish peroxidase-conjugated sheep anti-mouse Ig (Amersham). Immunoreactive proteins were detected by using the enhanced chemiluminescence detection system (Amersham) and autoradiography film.

NOS enzymatic activity assay. NOS activity was measured in terms of L-citrulline formation from L-arginine substrate. For each sample, 200 μg of tissue homogenate (see above) protein were incubated in a buffer (pH 7.4) containing 50 mM Tris·HCl, 2 mM $CaCl_2$, 10 μg/ml calmodulin, 1 mM NADPH, 10 μM FAD, 10 μM tetrahdrobiopterin, and 10 μM L-[^{3}H]arginine in a total volume of 100 μl, for 1 h at 37°C. The reaction was terminated by adding 100 μl cold buffer (pH 5.5) containing 100 mM HEPES, 10 mM EDTA, and 10 mM EGTA. One milliliter of Dowex AG50W-X8 (Sigma Chemical, 200–400 mesh, 8% cross-linked, Na^+ form; 1:1 wt/vol in water) was added to the buffer to remove excess L-[^{3}H]arginine. To determine the amount of L-citrulline formed, the sample was centrifuged for 20 min at 5,000 *g*, and the radioactivity in the supernatant was measured by a liquid scintillation counter (model DU6000, Beckman). The nonspecific radioactivity of enzymatic reaction was determined by adding N^G-nitro-L-arginine methyl ester (L-NAME; NOS inhibitor, 5 mM) into the buffer. Calcium-independent activity was measured using calcium/calmodulin-free buffer containing 1 mM EDTA plus 1 mM EGTA. The iNOS activity was then calculated by computing the difference between the EDTA+EGTA sample and L-NAME sample. The constitutive NOS (cNOS) activity was determined by computing the difference between the calcium/calmodulin sample and the EDTA+EGTA sample.

RT-PCR analysis. In this experiment, we analyzed mRNA levels in freshly harvested ECs sufficient in number for RT-PCR analysis. The cells were isolated from the mouse EDL skeletal muscle at 3 h after CLP by using a cell-trapping technique as described by our laboratory (44). EC identification was carried out as described by our laboratory (41). Isolated ECs were resolved in 1 ml TRIzol reagent (Invitrogen, Carlsbad, CA). RNA extraction was performed according to the procedures recommended by the TRIzol reagent manufacturer. A first-strand cDNA synthesis kit (Amersham) was used to synthesize cDNA by RT from RNA extracted from skeletal muscle ECs. The PCR reaction was performed by using a PCR Supermix kit (Invitrogen), following the procedures described by the manufacturer. The reactions were thermocycled 35 times (β-actin, 26 times) between 94°C (denaturation) for 1 min, 60°C (annealing) for 1 min, and 72°C (extension) for 1 min. The reactions were extended for an additional 7 min at 72°C after the last cycle was completed.

The primer pairs used were chosen from the published cDNA sequences of mouse endothelial NOS (eNOS) (18, Genbank accession no. NM_008713), mouse neuronal NOS (nNOS) (31, D14552), mouse iNOS (28, M84373), and human β-actin (5, X00351). All primer pairs spanned at least one intron in the genomic DNA sequence. The primers used for eNOS were 5′-CTG CTG CCC GAG ATA TCT TC-3′ (sense, bp 2,157–2,176) and 5′-CTG GTA CTG CAG TCC CTC CT-3′ (antisense, bp 2,386–2,367); the final PCR product was 230 bp in size. The primers used for nNOS were 5′-GGC ACC GGC ATC GCT CCC TT-3′ (sense, bp 3,846–3,865) and 5′-CTT TGG CCT GTC CGG TTC CC-3′ (antisense, bp 4,058–4,039); the final PCR product was 213 bp in size. The primers used for iNOS were 5′-AGC ATC ACC CCT GTG TTC CAC C-3′ (sense, bp 1,577–1,596) and 5′-TGG GAC AGT CTC CAT TCC CA-3′ (antisense, bp 1,964–1,945); the final PCR product was 388 bp in size. Oligonucleotide primers for β-actin (sense, 5′-AAC CGC GAG AAG ATG ACC CAG ATC ATG TTT-3′; and antisense, 5′-AGC AGC CGT GGC CAT CTC TTG CTC GAA GTC-3′) were used as a total RNA control in each sample, based on previous work by Briggs and coworkers (5). The PCR products were separated on a 1.5% agarose gel, stained with ethidium bromide, illuminated with ultraviolet light, and photographed.

The RT-PCR products for eNOS, nNOS, and iNOS were ligated into pCRII vector (Invitrogen), and the subsequent plasmid DNA was purified by using ion-exchange columns (Qiagen, Chatsworth, CA). To confirm the authenticity of the RT-PCR product, each insert was sequenced with ThermoSequenase using the dideoxynucleotide chain termination reaction (Amersham). The samples were resolved on a DNA sequencer (model 725, Molecular Dynamics).

Measurement of ascorbate. Ascorbate concentration in the EDL muscles, harvested from mice euthanized at 6 h after CLP, was assayed by acidic extraction and HPLC with elec-

trochemical detection, according to the procedures described previously by us (1). Ascorbate was quantified with a Waters M460 amperometric detector and the concentrations in the skeletal muscles were determined by interpolation on an external standard curve.

Measurement of oxidant levels. The oxidant-sensitive probe dihydrorhodamine 123 (DHR; Molecular Probes, Eugene, OR) was used to evaluate in vivo the production of ROS. This assay makes use of the fact that cell-permeable DHR reacts with ROS to form fluorescent rhodamine inside cells (35). For these experiments, mice were injected with a 0.1-ml bolus of DHR (1.5 μmol/kg) through the tail vein 4 h after the CLP procedure. The EDL muscles were harvested from mice killed at 2 h after DHR injection. The tissues from each animal were homogenized in 5 vol (wt/vol) of saline. Thereafter, an equal volume of 100% ethanol was added to the homogenate to precipitate proteins. The fluorescence intensity of rhodamine in the supernatant was then measured by fluorescence spectrophotometry at excitation and emission wavelengths of 502 and 523 nm, respectively. The oxidant level was expressed as fluorescence intensity per milligram tissue wet weight.

Blood pressure and arteriolar diameter measurements. Mice were anesthetized intraperitoneally with ketamine (90 mg/kg) plus xylazine (4 mg/kg) 6 h after CLP. The body temperature was maintained at 37°C by means of a heating pad. The carotid artery was cannulated for monitoring arterial pressure. The jugular vein was cannulated for administration of NE. Increasing doses of NE (0 to 500 ng/kg; dissolved in sterile 0.9% saline) were injected in 0.1-ml boluses. Sufficient time (i.e., 10 min) was allowed between injections to restore the arterial pressure after its temporary increase caused by the NE bolus. To measure arteriolar diameter, the EDL muscle was exposed and wetted with saline and then covered with degassed mineral oil and Saran wrap to prevent tissue drying. A coverslip was placed on the EDL muscle, its surface epi-illuminated by means of fiber-optic light guide, and its microcirculation viewed through a Leitz ELR microscope equipped with a ×20/0.32 numerical aperature objective and a ×10 eyepiece. The image was captured by a video camera, displayed on a monitor, and recorded on videotape. Arteriolar diameter was measured off-line from the video monitor by using a caliper (resolution ± 1.5 μm). In general, it was technically difficult to obtain sharp images of the arteriolar wall in the present epi-illuminated preparation. For this reason, in the majority of experiments, the diameter was measured in terms of the width of the red blood cell column in the arteriole. However, in some experiments, the quality of the arteriolar image was sufficient to measure both the width of the column and the luminal diameter on the basis of arteriolar wall-to-wall separation. In these experiments, we were able to verify that the width of the red blood cell column was the same as the luminal diameter. Baseline diameters of randomly selected terminal arterioles (i.e., vessels running obliquely to the direction of skeletal muscle fibers) ranged from 6 to 10 μm. Arteriolar vasoconstrictive reactivity was assessed by measuring the largest change in diameter at randomly preselected arteriolar sites after intravenous administration of NE as described above.

Data analysis. Data are expressed as means ± SE values. Statistical analysis was performed by one-way ANOVA followed by a two-tailed Student's *t*-test with Bonferroni correction for multiple comparisons. Probabilities of $P < 0.05$ were considered significant.

RESULTS

Ascorbate inhibits iNOS expression. As shown in Fig. 1*A*, plasma NOx levels rose by 6 h after CLP, remained elevated at 12 h, and returned to baseline by 24 h. The time course of NOx production thus suggested that CLP increased NO synthesis at the early stage of sepsis. Figure 1*B* shows that ascorbate alone did not influence NOx production in non-CLP mice; however, ascorbate administration blocked the NOx elevation in the plasma of CLP mice at 6 h after CLP.

We next determined the effect of ascorbate on the protein expression of all three NOS isoforms, and on the enzymatic activity of both cNOS and iNOS, within the EDL muscles of CLP mice. A 6-h CLP insult caused a clear iNOS induction in the skeletal muscle at both protein (Fig. 2, *A* and *B*) and activity (Fig. 2*C*) levels. Ascorbate administration completely blocked the iNOS induction but did not influence the cNOS (including eNOS and nNOS) protein (Fig. 2, *A* and *B*) and activity (Fig. 2*C*) expression pattern in the skeletal muscles of CLP mice.

We further determined the effect of ascorbate on the mRNA levels of all three NOS isoforms in ECs freshly harvested from EDL skeletal muscles of CLP mice. iNOS mRNA was induced in the skeletal muscle ECs at 3 h after CLP; this induction was completely blocked by prior ascorbate administration (Fig. 3, *A* and *B*). Neither CLP nor ascorbate administration plus CLP altered the eNOS mRNA transcription pattern (Fig. 3,

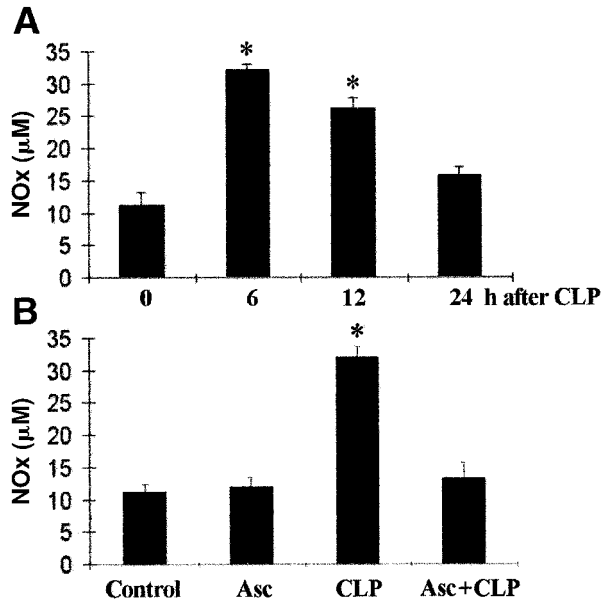

Fig. 1. *A*: cecal ligation and puncture (CLP) causes elevation of plasma levels of nitrite and nitrate (NOx). Blood samples were drawn at 0, 6, 12, and 24 h after CLP. Values are means ± SE for 6 mice. *$P < 0.05$ compared with 0 h after CLP. *B*: ascorbate administration to CLP mice prevents the elevation of plasma NOx levels. Mice were given an intravenous injection of 0.1 ml bolus of saline (control) or a bolus of ascorbate (Asc, 200 mg/kg body wt; 30 min before CLP), and blood samples were collected at 6.5 h after these injections. Values are means ± SE values for 6 mice. *$P < 0.05$ compared with control group.

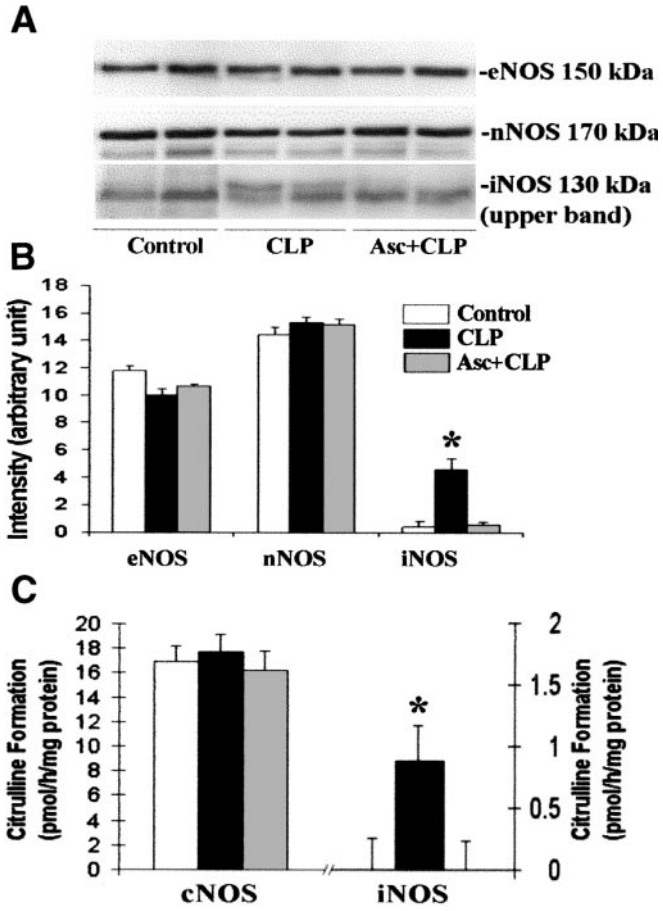

Fig. 2. Ascorbate administration prevents the induction of inducible nitric oxide synthase (iNOS) protein and enzymatic activity in CLP mice. Mice were treated as described in Fig. 1*B*. Extensor digitorum longus muscles were harvested 6 h after CLP. *A*: representative Western blot examples from 2 mice in each group (for iNOS, the upper band is specific and the lower band is nonspecific). *B* and *C*: summaries of NOS isoform band intensities and enzymatic activities, respectively, from 6 mice in each group (means ± SE). Constitutive NOS (cNOS) includes neuronal NOS (nNOS) and endotheial NOS (eNOS). *$P < 0.05$ compared with control and Asc+CLP groups. Ascorbate treatment did not influence the cNOS protein expression and activity in the skeletal muscle of CLP mice.

A and *B*). nNOS mRNA was not detectable in these ECs.

The results above indicate that ascorbate selectively inhibited the CLP-stimulated iNOS expression while at the same time maintaining the normal cNOS protein and activity levels. Furthermore, the RT-PCR analysis reveals that iNOS mRNA was induced in the microvascular ECs from the septic skeletal muscle and that ascorbate inhibitory effect occurred at the transcription level.

Ascorbate prevents CLP-stimulated oxidative stress. Ascorbate is a potent antioxidant that scavenges ROS, including superoxide anion and hydroxyl radicals (8, 30). We determined the effect of ascorbate on oxidant levels in the septic skeletal muscle. As shown in Fig. 4*A*, ascorbate administration significantly elevated the ascorbate concentration in the skeletal muscles of control and CLP mice. Moreover, ascorbate prevented the increase of oxidant levels in the skeletal muscle of CLP mice (Fig. 4*B*). These results indicate that ascorbate acts as an antioxidant in skeletal muscle exposed to septic insult.

Ascorbate preserves microvascular reactivity to catecholamines. CLP decreased the baseline arterial pressure (Fig. 5). Ascorbate administration partially prevented this decrease (Fig. 5). Our next experiments determined the effect of ascorbate on the vascular response to NE within the EDL muscle. As shown in Fig. 6, CLP impaired both arteriolar constriction (Fig. 6*A*) and arterial pressure (Fig. 6*B*) responses to NE. Ascorbate administration completely prevented these impairments. Ascorbate administration did not affect the changes in vascular diameter and arterial pressure caused by NE in non-CLP mice (data not shown). Together, these results indicate that ascorbate administration partially restored the baseline arterial pressure and completely preserved the microvascular constriction and arterial pressure responses to NE in CLP mice.

DISCUSSION

Oxidative stress occurs in skeletal muscle early after the onset of systemic microbial infection (27, 38). Using an oxidant-sensitive fluorescence probe, DHR, to determine the total oxidant levels in the EDL muscle, we showed that sepsis causes an increase of ROS generation at 6 h after CLP (Fig. 4*B*). However, the increased oxidative stress could also reflect a decreased supply of

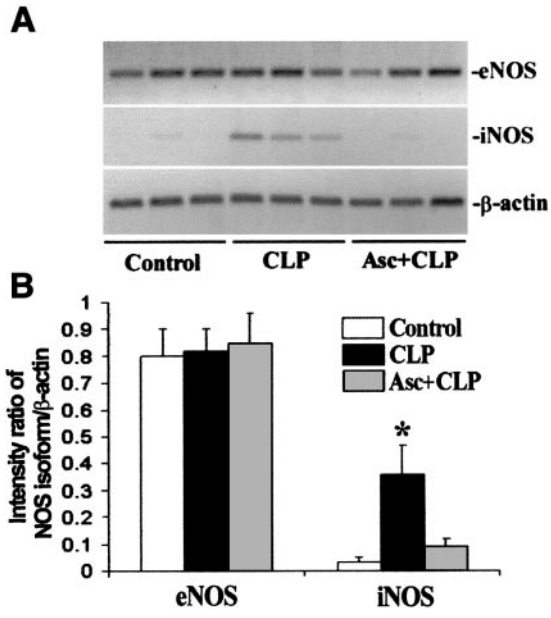

Fig. 3. Ascorbate administration prevents the induction of iNOS mRNA in septic skeletal muscle endothelial cells. Mice were treated as described in Fig. 1*B*. Microvascular endothelial cells were harvested from extensor digitorum longus skeletal muscles at 3 h after CLP. *A*: representative RT-PCR examples from 3 mice in each group (β-actin was included as total RNA control). *B*: summary of NOS isoform band intensities normalized to β-actin from 6 mice in each group (means ± SE). *$P < 0.05$ compared with control and Asc+CLP groups. nNOS mRNA was not detectable in the skeletal muscle endothelial cells.

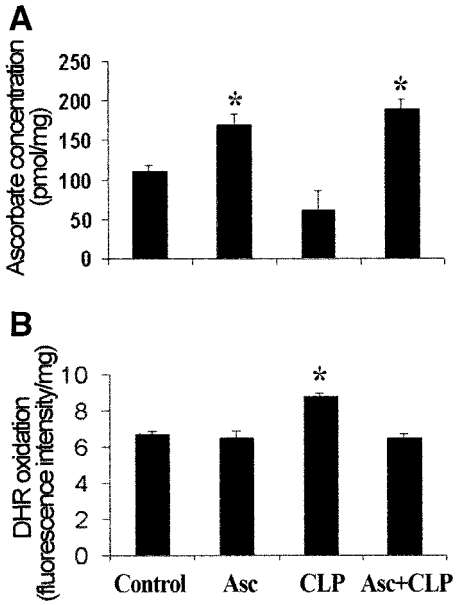

Fig. 4. *A*: ascorbate intravenous injection elevates ascorbate concentration within extensor digitorum longus muscle in control and septic mice (6 h after CLP). Mice were treated as described in Fig. 1*B*. Values are means ± SE for 6 mice. *B*: ascorbate administration prevents CLP-induced oxidative stress. Mice were treated as described in Fig. 1*B*. A 0.1-ml bolus intravenous injection of dihydrorhodamine 123 (DHR; 1.5 μmol/kg) was given 4 h after CLP. Muscles were harvested 2 h after DHR injection. Values are means ± SE for 6 mice. $^*P < 0.05$ compared with the control group (i.e., mice injected with saline vehicle only).

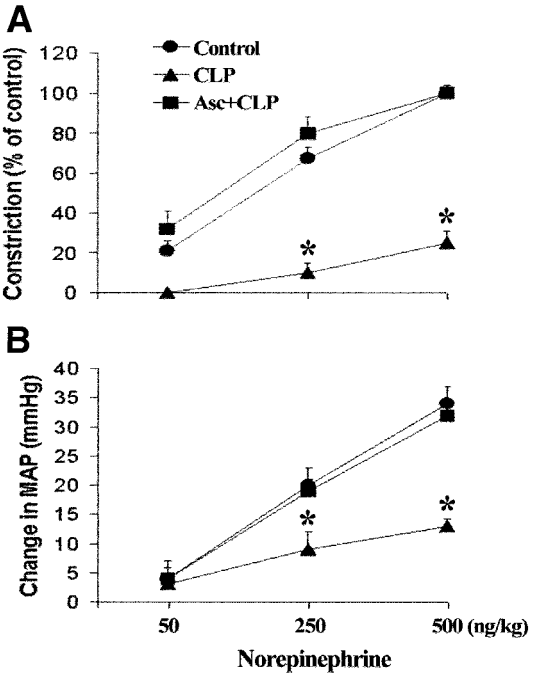

Fig. 6. Ascorbate administration restores the responses of vascular diameter (*A*) and MAP (*B*) to norepinephrine in mice at 6 h after CLP. Mice were treated as described in Fig. 1*B*. Norepinephrine was dissolved in sterile saline and given as a 0.1-ml bolus intravenous injection to anesthetized mice. Vasoconstrictive responses (%) are expressed as the diameter change divided by the baseline diameter. There were no observable responses to 0.1 ml bolus of saline (i.e., 0 ng/kg norepinephrine). The average baseline diameters before norepinephrine injection in control, CLP, and Asc+CLP mice were 7.5 ± 0.4, 8.5 ± 0.6, and 8.1 ± 0.6 μm, respectively. Values are means ± SE for 6 mice. $^*P < 0.05$ compared with the control group.

antioxidants. Indeed, it has been reported that endogenous ascorbate is depleted during sepsis (1, 4, 12). The present results also indicate that ascorbate concentration in the skeletal muscle tended to decrease at 6 h after CLP (Fig. 4*A*). This is likely because cellular mechanisms of ascorbate production are impaired in sepsis (23). Contrastively, ascorbate administration to the septic mice increased ascorbate concentration and abolished the CLP-stimulated oxidative stress within the skeletal muscle.

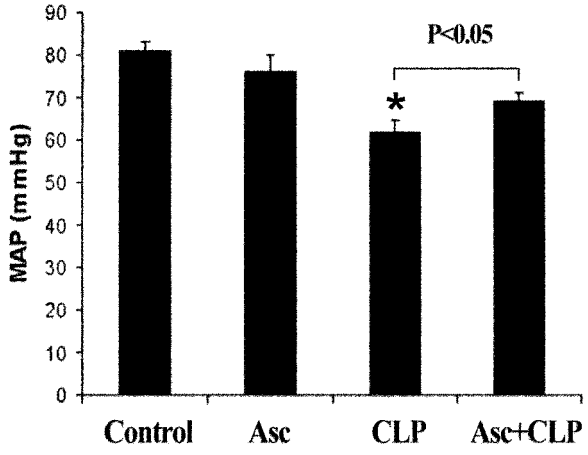

Fig. 5. Ascorbate intravenous injection partially prevents the decrease of arterial blood pressure [mean arterial pressure (MAP)] in septic mice (6 h after CLP). Mice were treated as described in Fig. 1*B*. Values are means ± SE for 8 mice in each group. $^*P < 0.05$ compared with the control group.

ROS, superoxide in particular, may act as initiating factors in the pathogenesis of sepsis (15, 47). When SOD, the superoxide anion scavenger, was given alone as a pretreatment, the blood pressure was maintained and survival was improved in septic rats (39) and mice (7). However, SOD had no effect on the blood pressure or survival when given to rabbits 1 h after infection with live *Escherichia coli* (6) or when given to rats after the onset of sepsis (40). It seems that SOD administration is effective only when given before the onset of sepsis (15). ROS could have mediated the systemic inflammatory response in sepsis, which ultimately leads to the expression of vascular iNOS (17). In the present studies, administration of ascorbate before sepsis decreased ROS levels and consequently attenuated iNOS expression. However, prior ascorbate administration only partially restored the decrease of baseline arterial pressure caused by CLP. The lack of complete restoration of baseline blood pressure by ascorbate could be due to possible iNOS-independent mechanisms of blood pressure regulation in sepsis or due to vasodilative ability of ascorbate itself. For instance, ascorbate has been reported to directly modulate the receptor- and voltage-operated Ca^{2+} channels, leading to vasodilation (9). In addition, a recent report

suggests that acidosis rather than excessive NO may cause the systemic hypotension during sepsis through reducing myocardial contractility (34).

The level of iNOS activity in septic skeletal muscle was much lower than that of cNOS (i.e., eNOS+nNOS; Fig. 2). Our data thus suggest that the iNOS expression in this tissue may be compartmentalized in the relatively small volume of vascular ECs and/or smooth muscle cells. To assess the possibility of induction of iNOS expression in ECs, we isolated ECs from the EDL skeletal muscles of CLP mice and conducted RT-PCR analysis of iNOS mRNA in these cells. The results demonstrated that CLP can induce iNOS mRNA transcription in the ECs of skeletal muscles and that this induction can be completely blocked by prior ascorbate administration (Fig. 3). Other investigators have reported that LPS induces iNOS expression in vivo in ECs of several tissues (26, 36). Our laboratory has shown that cultured ECs of rat EDL muscle origin express substantial iNOS on exposure to inflammatory stimulation (44). We have further demonstrated that iNOS expression in these cells is reduced by intracellular ascorbate in vitro (45). ECs play a pivotal role in the development of vascular dysfunction in sepsis (42). The present study demonstrated that ascorbate improvement of vascular reactivity could be due to protecting ECs from iNOS induction, which otherwise produces excessive NO.

It has been extensively documented that iNOS-derived NO contributes to the decreased systemic vascular resistance, impaired vascular responsiveness to catacholamines, and consequent hypotension present in sepsis (2, 21, 22, 33). However, the use of selective iNOS inhibitors for sepsis therapy is limited because they may also inhibit cNOS activity. For example, aminoguanidine was shown to prevent arterial pressure changes induced by LPS in rats (43). Further studies showed, however, that aminoguanidine enhanced LPS-induced intestinal vascular leakage due to inhibition of vascular cNOS activity (25). The present study showed that ascorbate injection selectively inhibited induction of iNOS by a septic insult. Furthermore, this was associated with complete prevention of vascular hyporeactivity and arterial pressure hyporesponsiveness to NE in CLP mice.

Ascorbate has been used to treat inflammatory symptoms of influenza and cold (14, 19). Injection of ascorbate also prevents skeletal muscle microvascular dysfunction in rats subjected to CLP (1). Our present data suggest that the anti-inflammatory property of ascorbate includes inhibition of iNOS expression. Additionally, ascorbate may preserve the activity of cNOS. Specifically, ascorbate maintains tetrahydrobiopterin, a critical cofactor for eNOS, in the reduced state (20). This effect of ascorbate is important in sepsis in maintaining the physiological functions of NO, such as preventing platelet and leukocyte adherence to endothelium (37). Another added benefit of therapy with ascorbate may be the protection of catecholamines from oxidation by superoxide (29). The inactivation of catecholamines by superoxide and NO has also been implicated in the pathogenesis of vascular hyporesponsiveness in sepsis (29). Because hospital infection constitutes a major cause of sepsis (10), acute administration of high-dose ascorbate along with antibiotic and supportive treatments before surgery, hemodialysis, or transplantation may reduce the morbidity and mortality associated with these interventions. Thus ascorbate could be used as an adjunct treatment in sepsis therapy.

In summary, the present data show for the first time that a bolus intravenous injection of ascorbate inhibits CLP-stimulated iNOS expression in mouse EDL muscle at both protein and activity levels. This effect of ascorbate is associated with reduced oxidative stress within the skeletal muscle. Our data suggest that ascorbate inhibition of iNOS expression contributes to the restored microvascular constriction and arterial pressure responses to NE.

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