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ScienceDirect
Free Radical Biology & Medicine 42 (2007) 124–131
www.elsevier.com/locate/freeradbiomed

Original Contribution

Ascorbate inhibits NADPH oxidase subunit p47phox expression in microvascular endothelial cells

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Received 7 June 2006; revised 25 September 2006; accepted 2 October 2006
Available online 6 October 2006

Abstract

The production of reactive oxygen species (ROS) is central to the etiology of endothelial dysfunction in sepsis. Endothelial cells respond to infection by activating NADPH oxidases that are sources of intracellular ROS and potential targets for therapeutic administration of antioxidants. Ascorbate is an antioxidant that accumulates in these cells and improves capillary blood flow, vascular reactivity, arterial blood pressure, and survival in experimental sepsis. Therefore, the present study tested the hypothesis that ascorbate regulates NADPH oxidases in microvascular endothelial cells exposed to septic insult. We observed that incubation with *Escherichia coli* lipopolysaccharide (LPS) and interferon-γ (IFNγ) increased NADPH oxidase activity and expression of the enzyme subunit p47phox in mouse microvascular endothelial cells of skeletal muscle origin. Pretreatment of the cells with ascorbate prevented these increases. Polyethylene glycol-conjugated catalase and selective inhibitors of Jak2 also abrogated induction of p47phox. Exogenous hydrogen peroxide induced p47phox expression that was prevented by pretreatment of the cells with ascorbate. LPS+IFNγ or hydrogen peroxide activated the Jak2/Stat1/IRF1 pathway and this effect was also inhibited by ascorbate. In conclusion, ascorbate blocks the stimulation by septic insult of redox-sensitive Jak2/Stat1/IRF1 signaling, p47phox expression, and NADPH oxidase activity in microvascular endothelial cells. Because endothelial NADPH oxidases produce ROS that can cause endothelial dysfunction, their inhibition by ascorbate may represent a new strategy for sepsis therapy.

Keywords: Ascorbate; Endothelial cells; Interferon-γ; Lipopolysaccharide; NADPH oxidase; p47phox; Sepsis

Introduction

Bacterial bloodstream infection exposes endothelial cells to blood-borne endotoxins (e.g., *Escherichia coli* lipopolysaccharide, LPS) and inflammatory cytokines (e.g., interferon-γ, IFNγ). The cells respond by activating NADPH oxidases that produce reactive oxygen species (ROS) and mediate alteration of cell function [1–3]. The enzyme activation occurs over the short term through phosphorylation of the enzyme subunits and over the long term by increased subunit expression [4–6].

The NADPH oxidase in phagocytes comprises a catalytic subunit Nox2 (i.e., gp91phox) and the regulatory subunits p22phox, p40phox, p47phox, and p67phox. Endothelial cells have been reported to contain these subunits, as well as the Nox2 homologs Nox1 and Nox4 [4–6]. Although the relative contribution of each Nox isoform to endothelial NADPH oxidase activity is uncertain [4–6], the p47phox subunit has been demonstrated to play an essential role in endothelial NADPH oxidase activation [7]. Further, p47phox-deficient endothelial cells show impaired production of reactive oxygen species when challenged by various stimuli [8–10].

Endothelial NADPH oxidases synthesize intracellular superoxide. Subsequent dismutation of superoxide to hydrogen

Abbreviations: EMSA, electrophoretic mobility shift assay; IFNγ, interferon-γ; iNOS, inducible nitric oxide synthase; L-NAME, N^G-nitro-L-arginine methyl ester; LPS, lipopolysaccharide; PEG-SOD, polyethylene glycol-conjugated superoxide dismutase; ROS, reactive oxygen species; SOD, superoxide dismutase.

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doi:10.1016/j.freeradbiomed.2006.10.033

peroxide may lead to prolonged redox signaling effects [11–13]. Excessive NADPH oxidase activity causes endothelial dysfunction. For instance, the endothelial barrier failure induced by an inflammatory cytokine is mediated by NADPH oxidase-derived ROS [14] and endothelial cells are killed by the high levels of ROS synthesized in these cells on exposure to bacteria [15]. Additionally, ROS produced by endothelial NADPH oxidases increase the expression of adhesion proteins and inducible nitric oxide synthase (iNOS), which may impair microvascular function in sepsis [4,16–18].

The production of ROS is central to the etiology of endothelial dysfunction in sepsis [1–3]. In particular, ROS have been implicated in the sepsis-induced impairment of capillary blood flow that is a strong predictor of death in patients with sepsis [19]. As sources of ROS, the NADPH oxidases in microvascular endothelial cells are potential targets for antioxidants that have been shown to improve outcome in experimental sepsis, e.g., ascorbate [20]. Ascorbate prevents LPS-induced hypotension [21], prolongs survival in experimental bacteremia [22], and improves capillary blood flow, vascular reactivity, arterial blood pressure, and survival in experimental sepsis [23–26]. This antioxidant is transported into microvascular endothelial cells and becomes concentrated intracellularly to millimolar levels [27,28]. Ascorbate scavenges superoxide and hydrogen peroxide, and it decreases oxidative stress in endothelial cells [27,28]. Intracellular ascorbate also alters gene expression and this effect appears to be mediated by redox-sensitive signaling pathways [29]. A better understanding of how these intracellular mechanisms operate after septic insult should provide an important foundation for developing novel therapies. Therefore, the aim of the present study was to test the hypothesis that ascorbate acts through redox-sensitive signaling pathways to modify the expression of NADPH oxidase subunit p47phox in microvascular endothelial cells exposed to septic insult.

Materials and methods

Cell cultures

The University of Western Ontario Council on Animal Care approved the procedures. Microvascular endothelial cells were isolated from hind limb skeletal muscle of male C57BL/6 mice (Jackson Laboratory) using a cell-trapping technique we described previously [18]. Briefly, muscle tissue was dissected out aseptically under a microscope and placed in phosphate-buffered saline containing 100 U/ml penicillin and 100 μg/ml streptomycin. The muscle tissue was then minced into small pieces using dissecting scissors and digested in a solution containing 1 mg/ml collagenase type II, 0.12 mg/ml dispase, 0.12 mg/ml trypsin, and 1.5 mg/ml bovine serum albumin, for 30 min at 37°C. The tissue digests were filtered through a sterile 100-μm nylon mesh. The cells were pelleted by centrifugation at 200*g* for 5 min, resuspended in serum-supplemented M199, and incubated with magnetic beads coated with *Griffonia simplicifolia* lectin. After incubation at room temperature for 20 min, the magnetic beads and bound cells were trapped with a magnet and washed three times with serum-supplemented M199. The beads and cells were then removed from the magnet and plated into a 35-mm culture dish.

Endothelial cells were maintained in a growth medium consisting of DMEM/F12, 10% heat-inactivated fetal bovine serum, 0.2 mM L-glutamine, 25 μg/ml endothelial cell growth supplement (ECGS, BD Biosciences), 100 U/ml penicillin, and 100 μg/ml streptomycin, in a standard CO_2 incubator. Endothelial phenotype identification was carried out by immunocytochemical staining for von Willebrand factor, uptake of acetylated low-density lipoprotein labeled with 1,1-dioctadecyl-3,3,3-tetramethylindocarbocyanine perchlorate, and binding of fluorescently labeled *G. simplicifolia* lectin I [27]. Experiments were performed using confluent monolayers (passages 3 through 8) originating from at least three different mice for each experiment.

Experimental procedures

Endothelial cells were incubated in a culture medium that consisted of growth medium from which ECGS was omitted. The cells were pretreated with ascorbate, hydrogen peroxide, or other drugs with the indicated doses for the indicated periods before exposure to septic insult [i.e., the combination of *E. coli* LPS (25 ng/ml) and IFNγ (100 U/ml)]. Except otherwise indicated, the cells were then washed twice with PBS, scrape-harvested in PBS, and then pelleted by centrifugation at 10,000*g* for 10 min. The cell pellets were stored at −80°C for further assays.

Superoxide production was measured using a previously described cytochrome *c* reduction assay [30] with minor modifications. Cells harvested from each 35-mm culture dish were disrupted by freeze-thawing in 100 μl lysis buffer (pH 7.4) composed of 5 mM potassium phosphate, 250 mM sucrose, 0.1 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. This mixture was added to 400 μl phenol red-free DMEM with 40 μM cytochrome *c* and 100 μM NADPH and was incubated at 37°C for 1 h with or without manganese superoxide dismutase (SOD, 500 U/ml). The reduction of cytochrome *c* was measured spectrophotometrically at 550 nm. Reduction of cytochrome *c* in the presence of SOD was subtracted from the values without SOD. The absorbance differences between comparable wells with and without SOD were converted to equivalent superoxide release by using the molecular extinction coefficient for cytochrome *c* of 21 mM^{-1} cm^{-1}. NADPH oxidase activity was expressed as nanomole superoxide per milligram protein per hour.

Intracellular ascorbate concentrations were determined by HPLC with electrochemical detection using a previously described method [27]. Cells in 12-well plates were washed twice with 1 ml/well of cold Tris buffer [containing 10 mM Tris-Cl (pH 7.3) and 320 mM sucrose], and then scraped into 0.25 ml of cold water. Aliquots of 50 μl were removed and frozen for protein determination, and aliquots of 180 μl were mixed with 20 μl of 8.5% metaphosphoric acid and frozen for ascorbate measurements. Samples were stored at −80°C for up to 1 week before analysis.

Western blot analysis was performed as follows. Cells harvested from each well of 12-well plates were lysed in