Ascorbate-mediated enhancement of reactive oxygen species generation from polymorphonuclear leukocytes: modulatory effect of nitric oxide

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Abstract: Recent studies from our laboratory have demonstrated that ascorbate potentiated enzymatic synthesis of nitric oxide (NO) from polymorphonuclear leukocytes (PMNs). NO is known to modulate various function of PMNs such as chemotaxis, adherence, aggregation, and generation of reactive oxygen species (ROS). The role of ascorbate in the PMN phagocytosis, ROS generation, and apoptosis was thus evaluated in the present study. Ascorbate and its oxidized and cell-permeable analog, dehydroascorbate (DHA), did not affect the phagocytosis but enhanced ROS generation and apoptosis following treatment with *Escherichia coli* or arachidonic acid. A detailed investigation on the DHA-mediated response indicated that inhibitors of DHA uptake, reduced nicotinamide adenine dinucleotide phosphate oxidase, NO synthase, or ROS scavengers attenuated ROS generation. In DHA-treated cells, enhanced generation of peroxynitrite was also observed; thus, ascorbate-mediated ROS and reactive nitrogen species generation might mediate cytotoxicity toward the ingested microbes and subsequently, augmented PMN apoptosis. Results of the present study have helped in delineating the role of ascorbate in the modulation of NO-mediated ROS generation from PMNs. *J. Leukoc. Biol.* 75: 1070–1078; 2004.

Key Words: *ascorbic acid · peroxynitrite · apoptosis · phagocytosis*

INTRODUCTION

Nitric oxide (NO), an immunomodulator molecule, regulates various functions of lymphocytes, macrophages, and polymorphonuclear leukocytes (PMNs). The role of NO is proposed in chemotaxis, adhesion to endothelium, aggregation, and in the PMN-mediated tissue damage [1]. PMNs synthesize superoxide anion ($O_2^{\cdot-}$) by the one-electron reduction of oxygen at the expense of reduced nicotinamide adenine dinucleotide phosphate (NADPH) [2]. Studies from this laboratory as well as from others have demonstrated that NO modulates reactive oxygen species (ROS) generation from PMNs [1, 3, 4]. In addition to ROS scavenging, NO attenuated NADPH oxidase activity [5] and inhibited ROS generation, and a NO-dependent increase in the intracellular calcium augmented ROS generation [4]. NO-mediated modulation of ROS generation has also been demonstrated in the conditions associated with the increased accumulation of NO, such as hypoxia-reoxygenation [1, 4, 6] and lipopolysaccharide treatment [7].

Conversely, interaction of NO with $O_2^{\cdot-}$ leads to the formation of cytotoxic species, peroxynitrite ($ONOO^-$) and hydroxyl radicals [8, 9]. PMNs store high amounts of L-ascorbic acid, and activated PMNs recycle ascorbic acid/dehydroascorbate (DHA) [10, 11]. The precise reason for storing such high concentrations of ascorbic acid was not defined clearly. Studies from our laboratory have recently shown for the first time that ascorbate increased the availability of NO from PMNs by stabilizing tetrahydrobiopterin [12, 13]. As ascorbate affects the generation of NO from PMNs, it was considered worthwhile to investigate the effect of ascorbate or its oxidized product DHA on PMN functions such as phagocytosis, apoptosis, ROS, and reactive nitrogen species (RNS) generation.

MATERIALS AND METHODS

Chemicals

7-Nitro indazole (7-NI) and trifluoperazine (TFP) were purchased from RBI (Natick, MA). Annexin V binding assay kit, purified Annexin V, monoclonal anti-rat CD11b–fluorescein isothiocyanate (FITC), anti-rat CD45–phycoerythrin (PE), anti-human CD15–FITC, and anti-human CD45–PE were purchased from Becton Dickinson (San Jose, CA), and all other chemicals were procured from Sigma Chemical Co. (St. Louis, MO).

Animals and humans

Sprague Dawley rats (male, 180–200 g) were obtained from the animal house of Central Drug Research Institute (Uttar Pradesh, India). They were housed in polypropylene cages and provided with chow pellets and water ad libitum. To ascertain the results obtained in rats, we performed one set of experiments in venous monkey blood obtained from the Rhesus monkeys housed in the institute's animal house. We also performed studies on human blood obtained from male, healthy volunteers. All subjects were nonfasted and normotensive with no evidence of anemia, renal, or hepatic dysfunction. None of the donors were using antioxidants, vitamins, or nutritional supplements for at least 4 weeks before the blood collection. Animal handling and all the experiments were conducted according to the ethical guidelines of the institute.

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Preparation of ascorbic acid and DHA acid solutions

Stock solution (10 mM) of L-ascorbic acid and DHA were prepared fresh in Milli Q water just before the use, pH of the solution was adjusted to 7.0 with 1 N NaOH, and dilutions of the stock solutions (ascorbate or DHA) were made in Hank's balanced salt solution [HBSS; composition, mM: sodium chloride (NaCl), 138; potassium chloride, 2.7; disodium hydrogen phosphate, 8.1; potassium dihydrogen phosphate, 1.5; diethylenetriamine penta acetic acid, 0.1; pH 7.4; ref. 12].

Isolation of PMNs

Rat blood was collected under ether anesthesia by cardiac puncture in sodium citrate (0.129 M, pH 6.5, 9:1 v/v). Human or monkey venous blood was also collected in sodium citrate (0.129 M, pH 6.5, 9:1 v/v). Platelet-rich plasma was removed by the centrifugation of blood at 250 *g* for 20 min at 20°C (Sigma Centrifuge, Germany). Buffy coat was collected after centrifugation of the remaining blood at 1000 *g* for 20 min at 20°C. The buffy coat was subjected to dextran sedimentation and Histopaque gradient at 700 *g* for 30 min at 20°C as described earlier [12]. PMNs were harvested at the interface of Histopaque 1119/1077 and were washed three times with HBSS. Cell viability was tested by trypan blue exclusion test. Purity of rat PMNs was checked by labeling with anti-rat CD11b–FITC and anti-rat CD45–PE and for human PMNs, with anti-human CD15–FITC and anti-human CD45–PE monoclonal antibodies, according to instructions provided by the manufacturer (Becton Dickinson) using a flow cytometer (FACSCalibur, Becton Dickinson), which was always more than 90%.

ROS generation

PMNs (2×10^6 cells/ml) were incubated for 5 min with 2′, 7′ dichlorodihydrofluorescein diacetate (DCDHF-DA; 10 μM) and were then treated with ascorbate or DHA for 15 min at 37°C. Arachidonic acid [AA; 2 μM; formyl-Met-Leu-Phe (fMLP), 5 μM] and phorbol 12-myristate 13-acetate (PMA; 30 nM) were used to induce the free radical generation, and cells were incubated further at 37°C for 15 min. In another set of experiments, PMNs (2×10^6 cells/ml) were preincubated for 5 min with DCDHF-DA (10 μM), and then ascorbate or DHA and bacteria ($1–2\times10^7$ cells/ml) were added to further incubate for 30 min at 37°C. Each sample was evaluated on a FACSCalibur (Becton Dickinson). Fluorescence of 10,000 cells was acquired by gating the PMN population and was analyzed by the Cell Quest program to determine the mean fluorescence. To determine the effect of various interventions, cells were incubated at 37°C with NADPH oxidase inhibitor diphenyleneiodonium (DPI) or NO synthase (NOS) inhibitor 7-NI for 30 min, NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), $ONOO^-$ scavenger mercaptoethylguanidine (MEG), or iodoacetamide for 15 min and for 5 min with ROS scavengers superoxide dismutase (SOD) and catalase, myeloperoxidase (MPO) inhibitor 4-aminobenzoicacid hydrazide (ABAH), calmodulin (CaM) antagonist TFP, or DHA transport inhibitor (D-glucose) before loading with the dye. The interventions used in the study and ascorbate/DHA were analyzed for their per se effect on the DCDHF-DA fluorescence in isolation or in combination on a fluorimeter (RF5000, Schimadzu, Japan). None of these interventions, alone or in combination with ascorbate/DHA, exhibited any significant effect on the fluorescence to DCDHF-DA. In one set of experiments, dihydroethidium (DHE; 10 μM) was used to assess the effect of ascorbate or DHA (300 μM) on the superoxide radical generation.

Generation of ROS from PMNs has been reported as mean stimulation index (MSI) as described earlier [7], which is the ratio of mean fluorescence of the stimulated and unstimulated cells. In all the experiments, MSI was calculated using respective control from the same experiment.

Phagocytosis

A flow cytometry-based method was used to study the bacterial phagocytosis by the rat and human PMNs as described previously [14]. Bacteria (*Escherichia coli*) were heat-inactivated at 60°C for 30 min before the labeling with FITC (50 μg/ml) in the dark with continuous stirring for 60 min at room temperature. Labeled bacteria were washed three times with HBSS, declumped, and resuspended in HBSS. PMNs (2×10^6 cells) were incubated with FITC-labeled bacteria ($1–2\times10^7$ cells/ml) for 30 min at 37°C in the presence or absence of ascorbate/DHA and were then washed and analyzed immediately by the FACSCalibur. To differentiate adherent from ingested, fluorescent bacteria, trypan blue (2 mg/ml for 5 min) was added to the samples after the first measurement on the FACSCalibur (Becton Dickinson), and the samples were analyzed again.

Annexin V binding

Annexin V FITC binding was performed according to the protocol of Becton Dickinson. Control PMNs (2×10^6cells/ml), ascorbate/DHA, bacteria (*E. coli*), and DHA-treated cells were incubated for 30 min at 37°C and were washed with cold HBSS. Cells were resuspended in 100 μl binding buffer [10 mM HEPES/NaOH (pH 7.5), 140 mM NaCl, 2.5 mM $CaCl_2$] and were incubated with 5 μl Annexin V FITC in the dark at room temperature [15]. After incubation, 400 μl binding buffer was added, and cells were subsequently analyzed by the FACSCalibur (Becton Dickinson). Specific binding of FITC–Annexin V was confirmed by incubating the PMNs with 10 μg blocking peptide (purified Annexin V) before incubation with FITC Annexin V as mentioned in the protocol.

Estimation of $ONOO^-$

Nitrate estimation

The Griess reagent measured nitrate content in the PMNs (1×10^7 cells). Cells following treatment with ascorbate/DHA were sonicated on ice and were centrifuged at 2000 *g* for 20 min at 4°C; thus, the supernatant obtained was stored at –20°C. Supernatants were freeze-dried and than reconstituted with 1 ml 0.7 M ammonium chloride. Each sample was divided into two fractions—one of them was treated with cadmium pellets so as to reduce the nitrate content to nitrite. Samples were treated with an equal amount of Griess reagent and incubated for 30 min at 37°C. Concentration of nitrite was estimated by measuring optical density at 548 nm using sodium nitrite as standard. Nitrite content in the samples not treated with cadmium was subtracted from total nitrite content (treated with cadmium) to obtain the nitrate content [16].

Estimation of 4-hydroxy-3-nitrophenylacetic (HPA) acid

HPA and its nitration product, 3-nitro-4-hydroxyphenyl acetic acid (3-nitro-HPA), were estimated in PMNs treated with ascorbate or DHA, according to Eiserich et al. [17] with minor modifications. Briefly, PMNs (3×10^7cells/ml in HBSS) were treated with HPA (1 mM) before the addition of ascorbate/DHA, and the reaction mixture was incubated at 37°C for 15 min. Subsequently, the cells were stimulated with AA (30 μM) for 15 min, and reaction was terminated by the addition of N-acetylcysteine (1 mM). The cell suspension was sonicated and centrifuged at 10,000 *g* for 20 min at 4°C, and the supernatant was passed through 10K cut-off filters. HPA and 3-nitroHPA in the filtrate were analyzed by high-pressure liquid chromatography (HPLC; LaChrom, Merck-Hitachi, Germany), using a LiChrospher 100 RP-18 column (5 μm, Merck-Hitachi), and 65% (50 mM) potassium phosphate (pH 3.0) and 35% methanol as mobile phase, using an ultraviolet detector at 274 nm. Identification and quantification of 3-nitroHPA and HPA were performed using external standards.

Detection of 3-nitrotyrosine

AA-stimulated PMNs treated with vehicle or DHA were harvested and fixed with 80% MeOH on ice for 20 min. Immunolabeling was performed in the fixed cells by incubating with a 1:200 dilution of polyclonal antibody to nitrotyrosine as primary antibody for 2 h. Cells were washed twice and stained with FITC-conjugated goat anti-rabbit immunoglobulin G for 1 h. Cells were washed three times before analysis by FACSCalibur (Becton Dickinson) [18]. AA-stimulated PMNs were labeled with secondary antibody alone and considered as controls.

Measurement of dihydrorhodamine 123 (DHR-123) fluorescence

PMNs (2×10^6 cells/ml) were preincubated for 5 min with DHR-123 (5 μM) and then treated with ascorbate or DHA for 15 min at 37°C before stimulation with AA (2 μM). Each sample was evaluated on a FACSCalibur (Becton Dickinson). Fluorescence of 10,000 cells was acquired by gating the PMN population and was analyzed by the Cell Quest program to determine the mean fluorescence [19].

Free radical generation from PMNs has been reported as MSI, as described earlier [7], which is the ratio of mean fluorescence of the stimulated and unstimulated cells. In all the experiments, MSI was calculated using respective control from the same experiment.

Statistical analysis

Results have been expressed as mean ± SE of at least five independent experiments in each group. Comparisons between two different groups were performed by unpaired Student's *t*-test or Welsh test, and multiple comparisons were made by one-way ANOVA followed by Newman Keul's test. Results were considered significant at $P < 0.05$.

RESULTS

Effect of ascorbate/DHA on bacterial phagocytosis and apoptosis

Phagocytosis of FITC-labeled *E. coli* by rat (**Fig. 1A**) or human PMNs (Fig. 1C) was not significantly altered in the presence of ascorbate or its cell-permeable analog DHA (300 μM; Fig. 1a), although ROS generation following phagocytosis was significantly enhanced (Fig. 1b). Annexin V binding to the PMNs following incubation with *E. coli* also was significantly augmented. In the presence of DHA, it was further increased. Blocking peptide, which was completely inhibited, assessed the specificity of Annexin V antibody binding (Fig. 1c). Similar trends of results were also observed in human PMNs (Fig. 1C, c).

Effect of ascorbate/DHA on ROS generation

A detailed investigation was undertaken to explore the effect of ascorbate/DHA on ROS generation in DCDHF-DA-loaded cells. Ascorbate or DHA did not interact directly with DCF to affect its fluorescence. Generation of ROS from PMNs was however significantly augmented in a time- and concentration-dependent manner by ascorbate/DHA (100 and 300 μM) in rat PMNs (**Fig. 2, a** and **b**). A similar increase in ROS generation was also observed in stimulated PMNs from monkeys and humans (Fig. 2, c and d, respectively).

AA (2 μM)-, fMLP (5 μM)-, or PMA (30 nM)-induced free radical generation was significantly enhanced in the presence of DHA (300 μM; **Fig. 3**). Pretreatment of PMNs with glucose (10 mM) or iodoacetamide (1 mM) to inhibit the DHA uptake

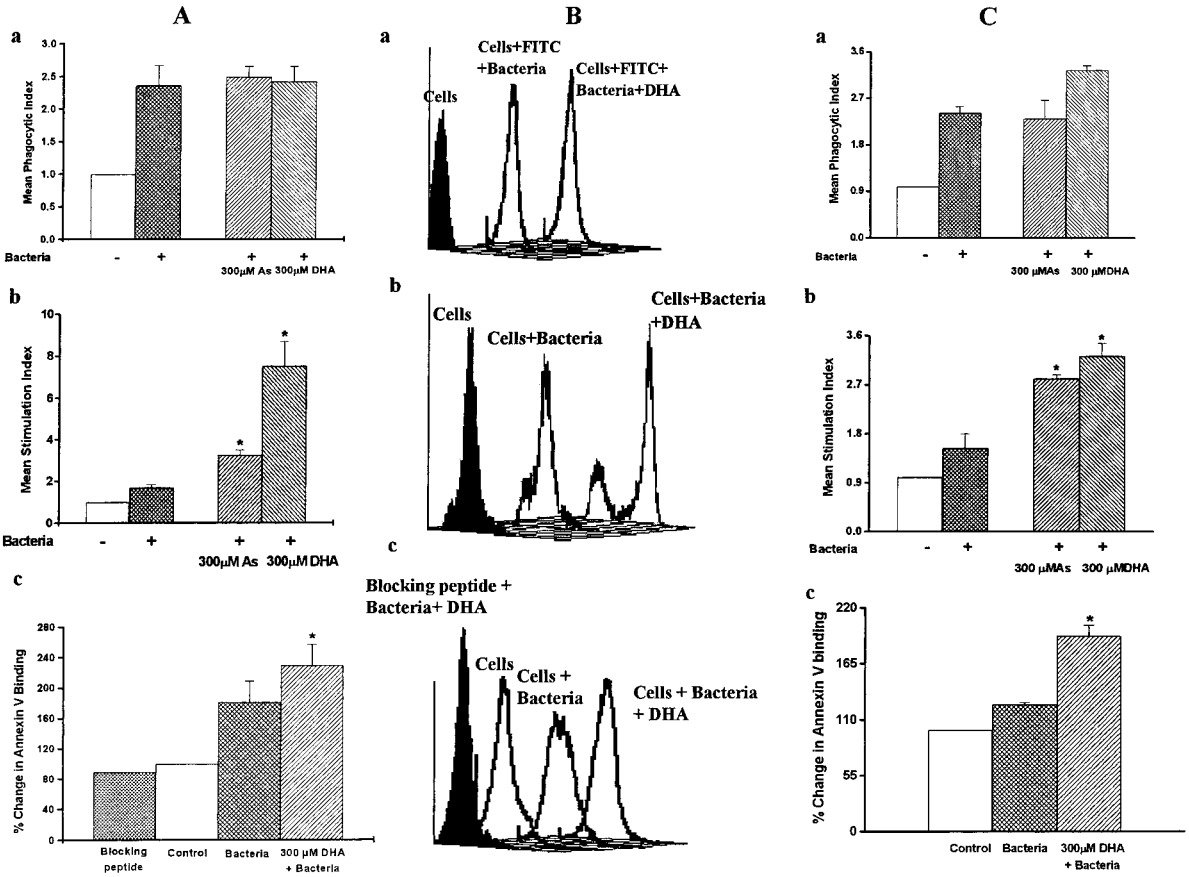

Fig. 1. Effect of ascorbate (As)/DHA on (a) phagocytosis, (b) ROS generation, and (c) apoptosis in the (A) rat and (C) human PMNs. A representative fluorescein-activated cell sorter (FACS) histogram showing phagocytosis, free radical generation, and apoptosis is given (B). (a) PMNs (2×10^6 cells) were incubated with FITC-labeled bacteria for 30 min at 37°C in the presence or absence of ascorbate/DHA and were then washed and analyzed immediately by FACS for assessment of phagocytosis. (b) For estimation of ROS generation, PMNs (2×10^6 cells/ml) were preincubated for 5 min with DCDHF-DA (10 μM) and then treated simultaneously with ascorbate/DHA and bacteria ($1–2\times10^7$cells/ml) for 30 min at 37°C and analyzed by flow cytometry. (c) Apoptosis was detected in PMNs (2×10^6 cells/ml) treated with bacteria and DHA for 30 min at 37°C and then washed with cold HBSS. Cells were resuspended in binding buffer, labeled with Annexin V FITC, and analyzed by flow cytometry. Data are expressed as mean ± SEM of at least three independent experiments in each group. *, $P < 0.05$, with respect to control in the presence of bacteria.

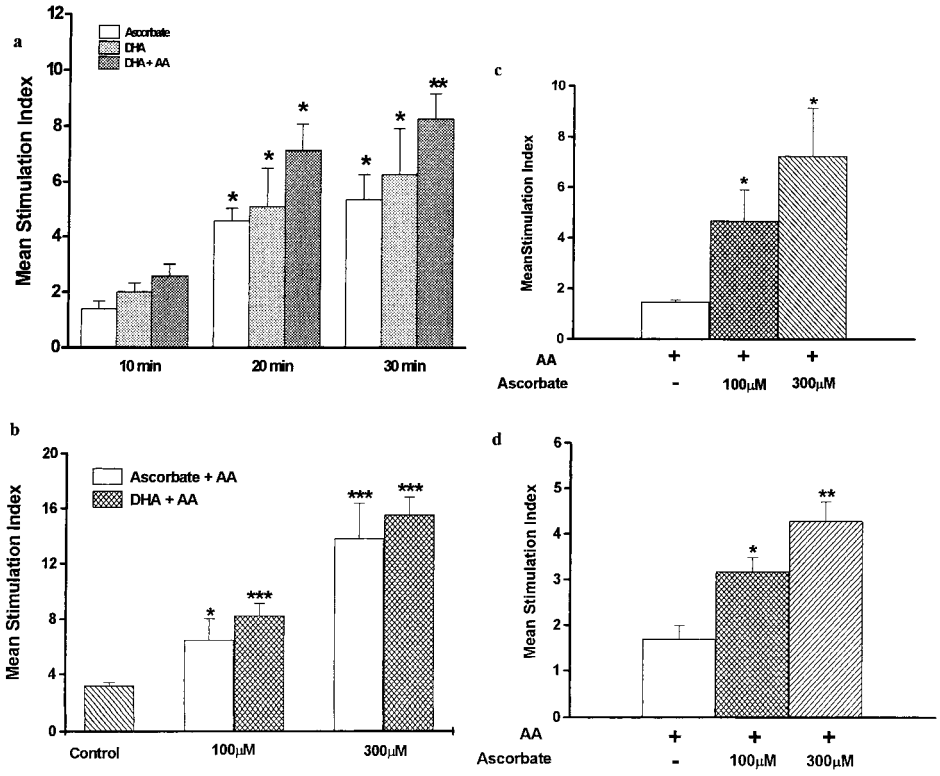

Fig. 2. Time- and concentration-dependent changes in ROS generation, as measured by flow cytometry using DCDHF-DA in PMNs treated with ascorbate/DHA. (a) Time-dependent increases in free radical generation following treatment of rat PMNs with 100 μM ascorbate or 100 μM DHA and 100 μM DHA + AA (2 μM). Data are expressed as mean ± SEM of at least five independent experiments. *, $P < 0.05$, ** $P < 0.01$, in comparison to free radical generation after 10 min of incubation. (b) Effect of ascorbate (100 and 300 μM) and DHA on AA induced free radical generation in rat PMNs. Data are expressed as mean ± SEM of at least 15 independent experiments. (c and d) Effect of 300 μM ascorbate on AA stimulated free radical generation in monkey and human PMNs, respectively. Data are expressed as mean ± SEM of at least three independent experiments in monkeys and three experiments in humans. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$, from stimulated cells.

or conversion of DHA to ascorbate, respectively, significantly reduced the increase in ROS generation from PMNs (**Fig. 4**).

Generation of ROS was also confirmed by using ROS scavengers, i.e., SOD (100 U/ml) and catalase (500 U/ml), which inhibited the increase in MSI (**Fig. 5**). Furthermore, ROS generation from DHA-treated cells or AA-stimulated PMNs pretreated with DHA (300 μM) was inhibited significantly on pretreatment with NADPH oxidase inhibitor DPI (10 μM) and CaM antagonist TFP (100 μM). Inhibitor of MPO, ABAH (100 μM), however, had no significant effect on the DHA response (Fig. 5). DHA and ascorbate at 300 μM concentration augmented the DHE fluorescence indication generation of superoxide radicals (**Fig. 6**).

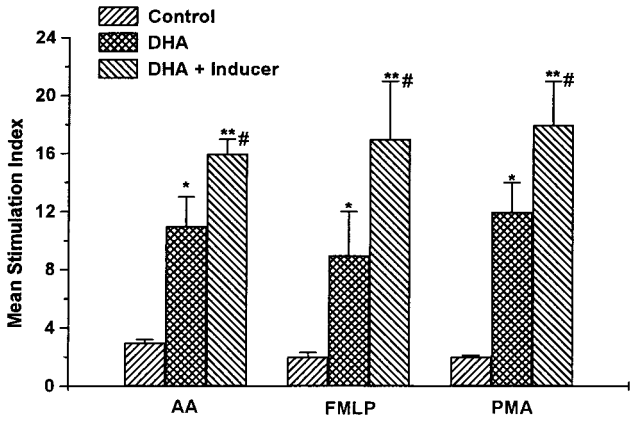

Fig. 3. Effect of DHA (300 μM) alone and on AA (2 μM)-, fMLP (5 μM)-, and PMA (30 nM)-induced free radical generation. *, $P < 0.05$, **,$P < 0.01$, with respect to the control; #, $P < 0.05$, in comparison with DHA (300 μM)-treated PMNs.

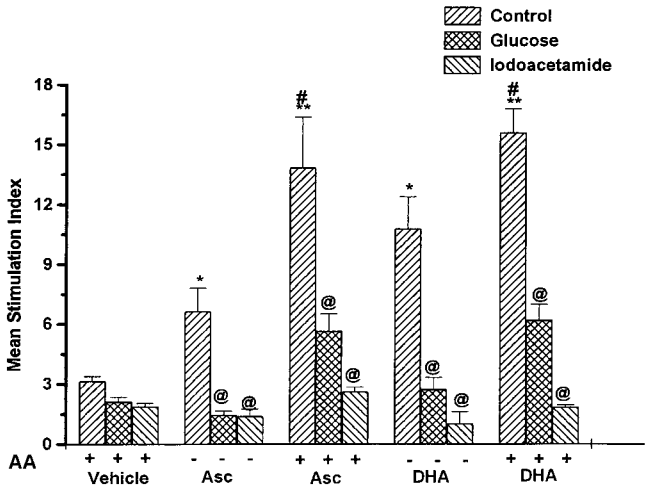

Fig. 4. Effect of inhibition of DHA transport or its conversion to ascorbate (Asc) on ROS generation measured by flow cytometry in DHA- and AA-stimulated rat PMNs (2×10^6 cells/ml), which were incubated with inhibitor of glucose transporter (GLUT)1, D-glucose (10 mM) for 5 min, or inhibitor of glutaredoxin, iodoacetamide (1 mM), for 15 min at 37°C before DCDHF-DA (10 μM) loading, addition of ascorbate, or DHA (300 μM), alone or with AA (2 μM). Data are represented as mean ± SEM of at least six independent experiments in each group. #, $P < 0.05$, in ascorbate or DHA-treated and AA-stimulated PMNs in comparison with ascorbate or DHA-treated PMNs; @, $P < 0.01$, in the glucose or iodoacetamide pretreated PMNs after ascorbate/DHA addition alone or with AA in comparison with the respective control. *, $P < 0.05$; **, $P < 0.01$, in comparison with the AA-treated control of the vehicle group.

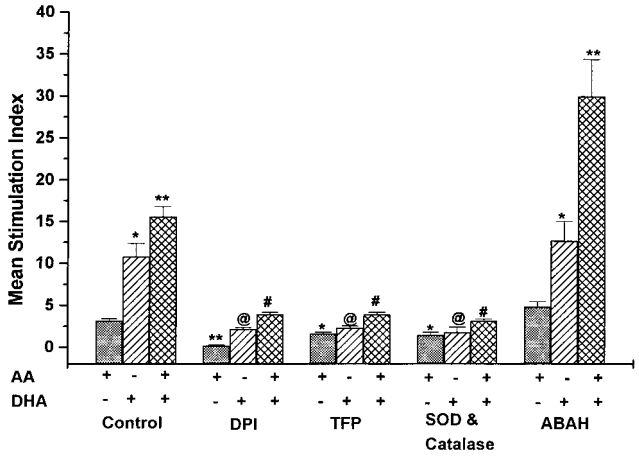

Fig. 5. Mechanism of augmented ROS generation on ascorbate/DHA treatment and in AA-stimulated PMNs. Effect of NADPH oxidase inhibitor, DPI (10 μM), CaM antagonist, TFP (100 μM), or scavengers of ROS, SOD (100 U/ml), and catalase (500 U/ml), MPO inhibitor, ABAH (100 μM), on DHA or DHA + AA-induced ROS generation using DCDHF-DA. Data are represented as mean ± SEM of at least four independent experiments. *, $P < 0.05$; **, $P < 0.01$, in AA-stimulated, receiving various treatments, in comparison with AA-stimulated PMNs receiving no treatment (HBSS). @, $P < 0.01$, in the presence of various interventions in comparison with DHA-treated control PMNs. #, $P < 0.01$, in the presence of various inhibitors/scavengers in comparison with DHA (300 μM) AA-treated, control PMNs.

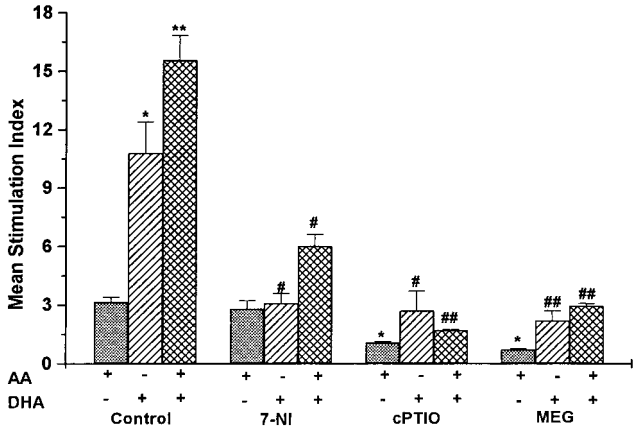

Fig. 7. Modulation of ROS generation on DHA-treated PMNs on AA stimulation by NO. Effect of NOS inhibitor 7-NI (1 mM, 30 min); NO scavenger cPTIO (30 μM, 15 min), and $ONOO^-$ scavenger MEG (100 μM, 15 min) on DHA- and AA (2 μM)-induced free radical generation using DCDHF-DA. Data are represented as mean ± SEM of at least three independent experiments in each group. *, $P < 0.01$; **, $P < 0.001$, in comparison with control, AA-stimulated cells. #, $P < 0.05$; ##, $P < 0.01$, in DHA-treated or DHA + AA-stimulated PMNs pretreated with inhibitor/scavenger compared with the respective controls.

Modulation of DHA-mediated ROS generation by NO interventions

Previous studies from our group have implicated the modulation of ROS generation from PMNs by NO [4, 6]. To investigate this phenomenon, PMNs were pretreated with a NOS inhibitor, 7-NI (1 mM), a NO scavenger, cPTIO (30 μM), or a scavenger of NO and $ONOO^-$, MEG (100 μM). None of these interventions attenuated the DCDHF-DA fluorescence in the resting PMNs. Inhibition of NOS with 7-NI attenuated the ROS generation in the DHA-treated cells or A-stimulated PMNs, and cPTIO and MEG inhibited the AA-induced stimulation completely, suggesting the involvement of NO in the PMN ROS generation (Fig. 6) [4]. Moreover, the scavengers of NO, $ONOO^-$, and inhibitors of NOS significantly attenuated the increase in ROS generation, also from DHA-treated PMNs (**Fig. 7**).

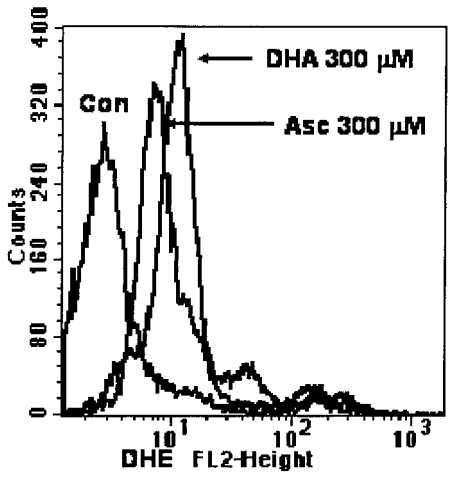

Fig. 6. Effect of ascorbate (Asc) or DHA (300 μM) on superoxide radical generation. For estimation of superoxide radical generation, PMNs (2×10^6 cells/ml) were preincubated for 5 min with DHE (10 μM) and then treated with ascorbate or DHA (300 μM).

Generation of $ONOO^-$ from PMNs treated with ascorbate/DHA

Nitrite accumulation correlates well with the formation of NO in cellular suspensions, and nitrate formation is considered to be a result of haemoglobin contamination with the generation of $ONOO^-$. PMNs treated with DHA (100 or 300 μM) showed a significant increase in the nitrate levels (**Fig. 8A**). Stimulation of PMNs with AA attenuated the nitrate content in control as well as DHA (300 μM)-treated PMNs. The nitrate content however, in AA-stimulated PMNs pretreated with DHA still remained significantly higher in comparison with the control (Fig. 8A).

$ONOO^-$ formation was assessed by 3-nitroHPA formation in activated PMNs in the presence of ascorbate or DHA. Formation of 3-nitroHPA was augmented in PMNs treated with ascorbate and AA, which was further enhanced in 300 μM DHA-pretreated, AA-stimulated cells (Fig. 8B).

$ONOO^-$ synthesis was also confirmed by nitrotyrosine antibody binding to the DHA-treated PMNs stimulated with AA by flow cytometry. Stimulated PMNs treated with DHA (300 μM) showed increased binding of antinitrotyrosine compared with stimulated PMNs alone (Fig. 8C).

Increase in the fluorescence of PMNs labeled with DHR-123 on treatment with 300 μM ascorbate or DHA also indicated augmented formation of $ONOO^-$ (Fig. 8D). Similar to nitrotyrosine antibody binding, fluorescence to AA + DHA-treated PMNs was higher compared with AA-treated cells.

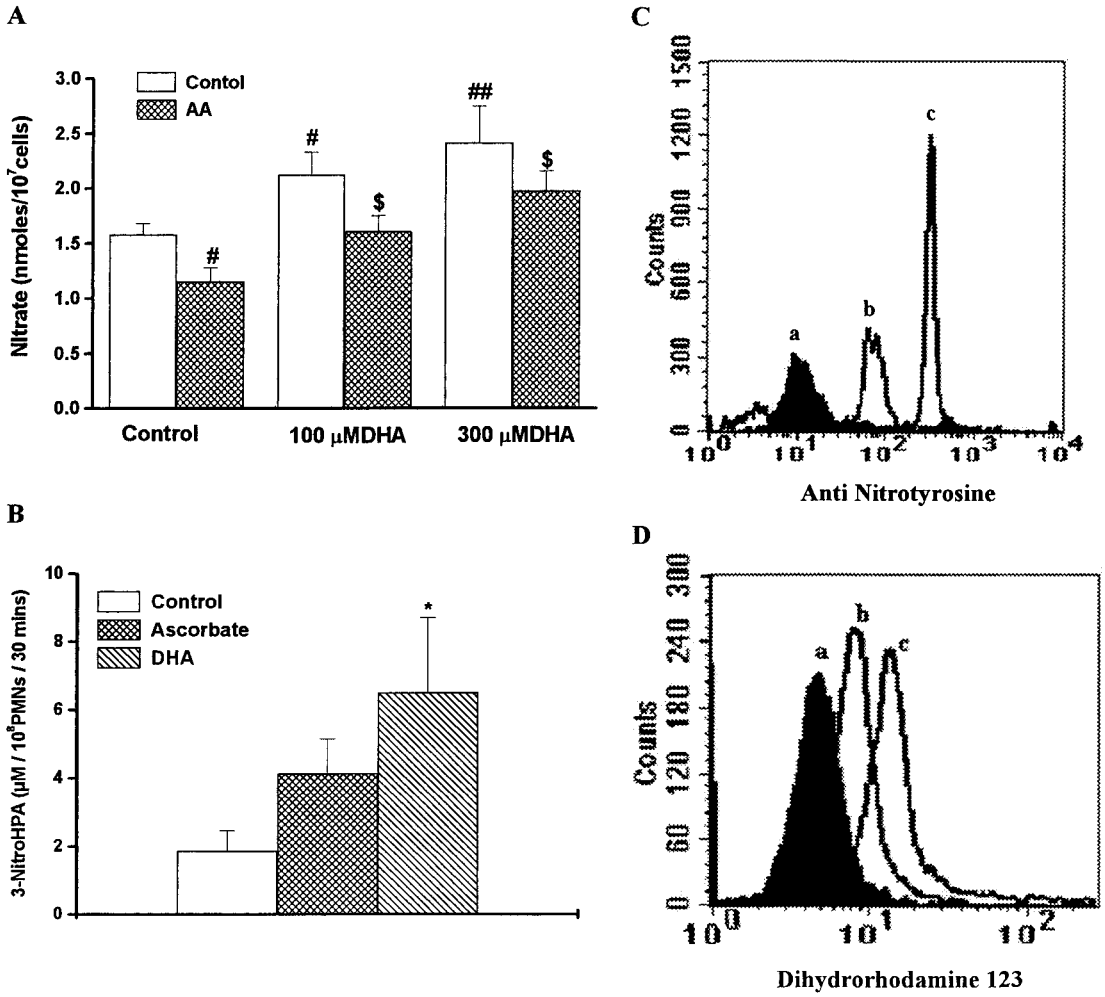

Fig. 8. Effect of ascorbate/DHA treatment to the AA-stimulated PMNs on $ONOO^-$ synthesis. (A) Nitrate content in rat PMNs (1×10^7cells/ml) as measured with Griess reagent. Data are represented as mean ± SEM of at least six independent experiments in each group. #, $P < 0.05$; ##, $P < 0.01$, from resting PMNs receiving no treatment (HBSS); $, $P < 0.05$, from stimulated control without DHA ($n=6-8$). (B) Formation of 3-nitroHPA on pretreatment of stimulated rat PMNs (3×10^7 cells) with HPA (1 mM) before reaction with 300 μM ascorbate/DHA as observed by HPLC. Concentrations of 3-nitroHPA were interpolated from an external standard method under the same conditions. Data are represented as mean ± SEM of at least 10 independent experiments in each group. *, $P < 0.05$, in DHA-treated cells compared with AA-stimulated controls. (C) Labeling of PMNs with antinitrotyrosine (a, FITC-labeled secondary antibody only; b, AA-treated cells labeled with primary and secondary antibody; and c, AA+DHA-treated cells labeled with primary and secondary antibody). (D) Oxidation of DHR-123 observed in FACSCalibur (a, control; b, 300 μM ascorbate+AA; c, 300 μM DHA+AA).

DISCUSSION

Results of the present study suggest that ascorbate-induced NO availability in the PMNs leads to the enhanced free radical generation and apoptosis, and phagocytic activity remained unaffected.

Neutrophils store ascorbic acid at ~10 mM concentration [10, 11, 20, 21]. Bacterial phagocytosis or chemical activation of PMNs has been shown to induce ascorbic acid recycling [11]. The precise role of ascorbic acid in such a high concentration in the neutrophil function remains less understood. In addition to its well-established role as an antioxidant and preserving NO [22], our recent studies have demonstrated that ascorbate also augments NOS activity [12, 13] by stabilizing tetrahydrobiopterin [12, 23–25]. NO is known to modulate various functions of PMNs including ROS generation [1].

In the present study, we evaluated the effect of ascorbate/DHA on bacterial phagocytosis, ROS, and RNS generation subsequent to phagocytosis and also on apoptosis to elucidate the functional importance of ascorbate-mediated augmentation of NO generation from PMNs. Bactericidal activity of the PMNs is associated with the activation of enzyme NADPH oxidase to generate ROS. The role of NO and $ONOO^-$ in bactericidal activity is also demonstrated [8, 9]. Bacterial phagocytosis by rat and human PMNs was not significantly altered in the presence of ascorbate/DHA (Fig. 1a). We, however, observed that free radical generation and binding of Annexin V antibody (Fig. 1, b and c), following bacterial phagocytosis, were augmented significantly in rat as well as human PMNs. It is therefore apparent that bacterial elimination in ascorbate/DHA-treated cells seems to be more efficient as a result of enhanced free radical generation; cells also readily undergo apoptosis to prevent inflammatory response. An ascorbate-induced increase in lipid peroxidation and proapoptotic death has been reported in different cell types [26–29]. It seems that the ascorbate pool in the PMNs is used by the PMNs to destroy

the engulfed pathogens, as suggested by our results and also by earlier observations that dietary supplementation with vitamin C augments innate immunity [30–33].

Studies on the effect of ascorbate on ROS generation from phagocytes are very limited, and the outcome has also remained controversial. These studies have shown increase [34–36], decrease [37], and even no change [30]. Previous studies from our laboratory have demonstrated modulation of ROS generation from the stimulated PMNs by NO [4, 6, 38, 39]. Ascorbate and its oxidized product, DHA, modulated the generation of NO from the PMNs [12, 13]. In the present study, we evaluated the modulatory role of ascorbate-mediated NO on the ROS generation from *E. coli* and AA-, fMLP-, and PMA-stimulated PMNs. As free radical generation was measured by flow cytometry, to elucidate the mechanism involved in ascorbate/DHA-mediated, enhanced free radical generation, we used a soluble and direct activator of NADPH oxidase, AA [40]. DCDHF-DA, the dye used in the present study to assess free radical generation, has been shown to react readily with H_2O_2 but also exhibits some reactivity with $ONOO^-$ [40, 41].

Ascorbate is oxidized readily to DHA in the aerated buffer and is subsequently transported into the PMNs by the GLUT1 transporter, which is further enhanced in activated PMNs [42]. Ascorbate/DHA elicited a time-dependent increase in the ROS generation even from resting cells (Fig. 2a), which was further enhanced on stimulation of rat PMNs with AA (Fig. 2b). The increase in ROS generation on ascorbate addition was also seen in PMNs obtained from monkey and human (Fig. 2, c and d, respectively). Inhibition of DHA uptake of by D-glucose [42] or conversion of DHA to ascorbate by iodoacetamide [12] reduced ROS generation (Fig. 4). Iodoacetamide is shown to inhibit a DHA-mediated increase in NO generation [12]. The enhanced ROS generation from AA-stimulated PMNs on DHA (300 μM) treatment was sensitive to DPI, a NADPH oxidase inhibitor, as well as to scavengers of ROS, i.e., SOD and catalase (Fig. 5), and ABAH, a MPO inhibitor, had no effect. DPI interferes with the flavin adenine dinucleotide binding to the enzyme NADPH oxidase and can also inhibit NOS [43]. Results obtained thus suggest that the increase in ROS generation was mediated by NO and was a result of the enzymatic synthesis of ROS.

Involvement of NO was also indicated by the inhibition of ROS generation from AA-stimulated PMNs on DHA (300 μM) treatment by a NOS inhibitor, 7-NI, or NO scavenger, cPTIO (Fig. 7). In the AA-stimulated PMNs, inhibition of ROS generation in the presence of NO scavenger implicates the involvement of NO in ROS generation. A biphasic role of NO has been demonstrated on the free radical generation from the PMNs [3, 4, 6, 38, 44]. Low or physiological concentrations (~10–100 nM) of NO potentiated ROS generation, but higher concentrations attenuated ROS generation from the rat PMNs [4]. The results from our earlier studies have indicated that treatment of PMNs with ascorbate led to the increase in NO generation [12, 13]. NO-mediated modulation of ROS generation from the PMNs indicates the possible role of ascorbate, which was not demonstrated earlier. To our knowledge, the present study is the first report of ascorbate-mediated augmentation of NO-dependent ROS generation.

Formation of NO and ROS on treatment of PMNs with ascorbate or DHA necessitated the estimation of $ONOO^-$; its formation in PMNs was confirmed by using various methods such as nitrate content, HPA acid estimation by HPLC, nitrotyrosine antibody binding, or DHR-123 fluorescence measurement by flow cytometry (Fig. 8). $ONOO^-$, a powerful oxidant, reacts with protein and nonprotein thiol residues [45], inhibits some enzymes of the mitochondrial electron transport chain [46], promotes lipid peroxidation [47], and finally, leads to mitochondrial permeability transition and apoptosis [46, 48]. Although ascorbic acid has been shown to attenuate the deleterious effect of this oxidant [49], our study exhibited that in PMN ascorbate, enhanced AA and *E. coli* induced ROS generation, as also observed by Guidarelli et al. [50] in U937 cells. It seems that supplementation of ascorbate in vitro to the PMNs increased the oxidative and nitrosative stress, which might be used by PMNs for effective bactericidal activity. Ascorbic acid has also been reported to act as a pro-oxidant under inflammatory conditions and increases nitrosative stress [2, 6, 50–52].

Circulating concentrations of ascorbate in normal, healthy individuals is in the micromolar range (50–200 μM), DHA concentration is several-fold less than ascorbate [20], and these concentrations exert a negligible effect on ROS generation. Ascorbate consumption and DHA accumulation observed during prolonged oxidative stress or pathophysiological states are characterized by a dominancy of pro-oxidant effects (e.g., diabetes mellitus) [53, 54]. DHA accumulation might be responsible, at least partly, for the oxidative stress associated with these pathologies. On the basis of the present study, it is however difficult to interpret that ascorbate per se might have proinflammatory effects, as ascorbate oxidation in plasma will be minimal, and ascorbate/DHA also promotes apoptosis. To investigate further the importance of ascorbate-mediated regulation of ROS generation, studies on scorbutic guinea pigs will be useful and are being pursued in our laboratory.

The present study demonstrated that ascorbate plays a significant role in the regulation of PMN functions. Activation of NADPH oxidase following *E. coli* or AA treatment in the presence of ascorbate/DHA augmented the ROS generation, which was sensitive to NOS inhibition. Increased ROS and NO availability also enhanced $ONOO^-$ formation, which in part, might contribute to the cytotoxicity subsequent to phagocytosis and initiates the apoptotic cascade in these cells. Results of the present study have helped systematically to delineate the physiological importance of ascorbate in microbicidal activity of PMNs by the enhanced free radical generation and apoptosis. Thus, NO seems to play a significant role in the ascorbate-mediated modulation of PMN function.

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