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Role of ascorbate in the regulation of nitric oxide generation by polymorphonuclear leukocytes

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Abstract

We have recently demonstrated that NO-mediated polymorphonuclear (PMN)-dependent inhibition of rat platelet aggregation is significantly enhanced in the presence of ascorbate. Consequently, the present study was undertaken to elucidate the underlying mechanisms involved in ascorbate-mediated potentiation of NO synthesis in PMNs. We observed that ascorbate or its oxidized product, dehydroascorbate (DHA), enhanced NOS activity, as measured by nitrite content, diaminofluorescein fluorescence or conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline in rat, monkey, and human PMNs. The increase in NO generation following ascorbate treatment was due to the intracellular ascorbate as iodoacetamide-mediated inhibition of DHA to ascorbate conversion attenuated the DHA-mediated increase in NO synthesis. The augmentation of NOS activity in the PMN homogenate by tetrahydrobiopterin was significantly enhanced by ascorbate, while ascorbate alone did not influence the NOS activity. Ascorbate-mediated enhancement of NOS activity in the cultured PMNs was significantly reduced in the presence of biopterin synthesis inhibitors. Ascorbate, thus, seems to regulate the NOS activity in the PMNs through tetrahydrobiopterin.

Keywords: Polymorphonuclear leukocytes; Nitric oxide; Tetrahydrobiopterin; Ascorbic acid; Dehydroascorbic acid

L-Ascorbic acid exerts multiple beneficial effects on cardiovascular disorders associated with impaired nitric oxide (NO)/cGMP signaling. In endothelial cells, ascorbate acts intracellularly [1] and could either improve the availability of tetrahydrobiopterin (BH_4) for NO synthesis [2] or spare intracellular thiols [3,4]. Recently, we also demonstrated the importance of ascorbate in regulating vascular hemostasis [5]. Addition of ascorbate to the polymorphonuclear leukocyte (PMN) suspension augmented NO release, which enhanced the inhibitory effect of PMNs on platelets. However, mechanism underlying the enhanced NOS synthesis by PMNs in the presence of ascorbate was not explored. NO seems to modulate various important functions of PMNs such as free radical generation, migration, apoptosis, and bactericidal activity [6–9]. However, the regulation of NOS activity in the PMNs is not very well understood.

Ascorbate increases NOS activity in the endothelial cells by stabilization of tetrahydrobiopterin [10]. In activated macrophages, ascorbate enhanced NO production through the iNOS pathway [11]. Ascorbate could preserve NO by reducing nitrite (NO_2^-) to NO [12,13]. However, there are no studies demonstrating that ascorbate can reduce nitrite to NO at physiologic pH in cells or in plasma. Like the endothelial cells, PMNs also accumulate high concentrations (~10 mM) of ascorbic acid. Activated PMNs release the oxidized product of ascorbate, dehydroascorbic acid (DHA), most of which is immediately taken up in PMNs by the glucose transporter (GLUT1) [14–16]. Once inside the cell DHA is reduced back to ascorbate by glutathione-dependent protein, glutaredoxin [17], and is stored in the cells in the reduced form. Kinetics of ascorbate and DHA uptake is well defined in the PMNs [14,16,17]. The physiological relevance of storing such a high concentration in the PMNs is however not well defined so far, though it has been repeatedly postulated that PMNs store high concentrations of ascorbate to defend themselves against oxidative injury [14,16,17].

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The present study was therefore undertaken in the PMNs to delineate the underlying mechanism(s) involved in ascorbate-mediated increase in NO generation.

Materials and methods

Chemicals. 7-Nitroindazole (7-NI) and trifluoperazine (TFP) were purchased from RBI. Anti-rat CD11b-FITC (Fluorescein isothiocyanate), anti-rat CD45-PE, and anti-human CD15-FITC, and anti-human CD45-PE were purchased from Becton–Dickinson (USA). Radioactive L-[^{3}H]arginine was obtained from Amersham, (UK), while all other chemicals were procured from Sigma Chemicals (USA).

Animals and humans. Sprague–Dawley rats (male, 180–200 g) were obtained from the animal house of Central Drug Research Institute. 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 non-fasted 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 prior to the blood collection. Animal handling and all the experiments were conducted according to the ethical guidelines of the institute.

Preparation of ascorbic acid and dehydroascorbic acid solutions. Ten millimolar stock solutions of L-ascorbic acid and DHA were prepared fresh in Milli Q water just before the experiment, pH of the solution was adjusted to 7.0 with 1 N NaOH, and dilutions of the ascorbate or DHA stock solutions were made in Hanks' Balanced Salt Solution (HBSS) (Composition, mM: sodium chloride (NaCl), 138; potassium chloride (KCl), 2.7; disodium hydrogen phosphate (Na_2HPO_4), 8.1; potassium dihydrogen phosphate (KH_2PO_4), 1.5; and diethylene triaminepentaacetic acid (DTPA) 0.1, pH 7.4).

Isolation of PMNs. Rat blood was collected under ether anesthesia by cardiac puncture in sodium citrate (0.129 M, pH 6.5, and 9:1 v/v). Venous blood from Rhesus monkeys and human was also collected in sodium citrate. The buffy coat was subjected to dextran sedimentation and Histopaque gradient at 700*g* for 30 min at 20 °C as described earlier [18]. PMNs were harvested at the interface of Histopaque 1119/1077 and washed three times with HBSS. Cell viability was tested by trypan blue exclusion test and was never less than 95%. Purity of rat PMNs was checked by labeling with anti-rat CD11b-FITC, anti-rat CD45-PE, and for human PMNs with anti-human CD15-FITC, anti-human CD45-PE monoclonal antibodies according to instructions provided by manufacturer (Becton–Dickinson, USA) using flow cytometer (FACS Calibur, Becton–Dickinson, USA), and was always more than 90%.

Nitrite estimation. Nitrite content in the PMNs (1×10^7 cells) was measured by the Griess reagent [18]. Cells following treatment with ascorbate/DHA were sonicated on ice and centrifuged at 2000*g* for 20 min at 4 °C, the supernatant thus obtained was stored at −20 °C.

Supernatants were freeze-dried and then reconstituted with 1 ml of 0.7 M ammonium chloride (NH_4Cl). Each sample was treated with cadmium pellets. Samples were treated with Griess reagent and incubated for 30 min at 37 °C. Concentration of nitrite was estimated by measuring the absorbance at 548 nm using sodium nitrite as standard.

Measurement of L-[^{3}H]citrulline synthesis in PMNs. NO production was assessed by the formation of L-[^{3}H]citrulline from L-[^{3}H]arginine. PMNs (1×10^7 cells) were suspended in incubation buffer (Composition, mM: Hepes 25; NaCl 140; KCl 5.4; $MgCl_2$ 1; and pH 7.4) along with 100 or 300 μM of ascorbate/DHA. Reaction was initiated by the addition of L-[^{3}H]arginine (0.16 μM) at 37 °C for 30 min and reaction was stopped by addition of ice-cold stop buffer (Composition, mM: NaCl, 118; KCl, 4.7; KH_2PO_4, 1.18; $NaHCO_3$, 1; EDTA, 4; N^{ω}-Nitro-L-arginine methyl ester (L-NAME), 2; and pH 5.5). Cells were sonicated (Heat Systems-Ultrasonic, INC) and the mixture was passed through Dowex (Na^+) columns. Radioactivity in the eluent was measured using β-scintillation counter (LKB Wallace, Liquid Scintillation counter) as described by Sethi et al. [19]. NO synthesis in the PMNs has been reported as pmol of [^{3}H]citrulline formed/30 min/10^7 cells.

To investigate the role of tetrahydrobiopterin (BH_4) in NOS activity, rat PMNs were sonicated and homogenized and then incubated with cofactors, NADPH, FAD, FMN, and calmodulin [18]. After incubation, BH_4 (10 μM), ascorbate (300 μM) or both were added and subsequently L-[^{3}H]arginine was added and NOS activity was estimated as described [19].

To confirm the contribution of tetrahydrobiopterin (BH_4) to the ascorbate-induced changes in NOS activity, rat PMNs were harvested and cultured in minimum essential medium (MEM) supplemented with 15% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin for 48 h in the presence or absence of BH_4 synthesis inhibitors (diamino-6-hydroxypyrimidine [DAHP], 5 mM and *N*-acetyl serotonin [NAS], 5 mM) in a 6-well plate. Cells were washed twice with HBSS and resuspended in incubation buffer to measure the NO synthesis in the presence and absence of 100 or 300 μM ascorbate/DHA as described above. Viability of the cultured cells was ~85% by the trypan blue exclusion test.

NO measurement by flow cytometry. PMNs (2×10^6 cells/ml) were incubated with 4,5-diaminofluorescein diacetate (DAF-2DA, 10 μM) for 5 min and then treated with ascorbate or DHA (100 or 300 μM) for 30 min at 37 °C. DAF-2DA reacts rapidly and irreversibly with NO to produce a highly reactive fluorescent product triazolofluorescein [20] (DAF-2T). Fluorescence of 10,000 cells was acquired by gating the PMN population and analyzed by the Cell Quest program to determine the mean fluorescence using FACS Calibur. To investigate the effect of various interventions, cells were incubated at 37 °C with iodoacetamide or 7-NI for 30 min, while, NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) and peroxynitrite scavenger mercaptoethylguanidine (MEG) were added just before loading with the dye.

NO generation from PMNs has been reported as the ratio of mean fluorescence (FL1) of control and treated cells [20] and represented as FL1 ratio. In all the experiments, the ratio was calculated using respective control from the same experiment.

Statistical analysis. Results have been expressed as means ± SEM of at least 5 independent experiments in each group. Comparison between two groups was performed by unpaired Student's "*t*" test and multiple comparisons were made by one-way ANOVA followed by Newman–Keul's test using GraphPad Prism 3.0 and Instat 2.0. Results were considered significant at $P < 0.05$.

Results

NO_x level and NOS activity in the presence of ascorbate/DHA

Nitrite content in the rat PMNs was significantly increased in the presence of 100 and 300 μM ascorbate or DHA (Fig. 1). Rise in the nitrite levels after DHA or ascorbate treatment was almost similar.

Synthesis of NO from DHA treated cells was also assessed by using L-[^{3}H]arginine. NOS activity was significantly augmented in rat PMNs following treatment with DHA in a concentration-dependent (100 and 300 μM) manner (Fig. 2A). Similar increase in the NOS activity on treatment with DHA (300 μM) was also observed in PMNs obtained from monkeys (Fig. 2B).

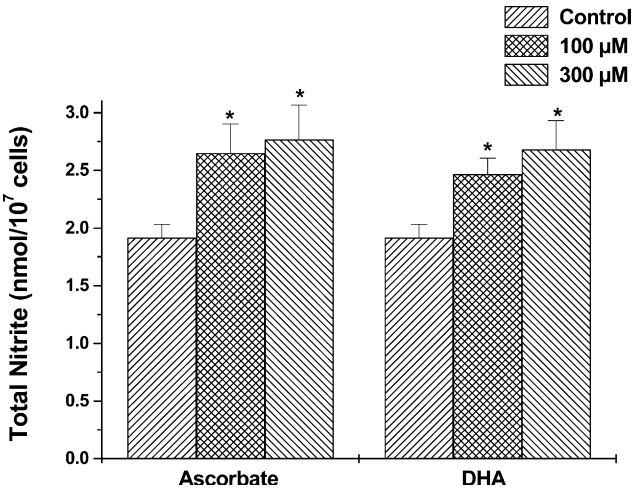

Fig. 1. Bar diagram representing total nitrite content from rat PMNs treated with vehicle, ascorbate or dehydroascorbate (DHA) (100 or 300 μM). Data are represented as means ± SEM of at least five independent experiments. $*P < 0.05$ in the ascorbate or DHA treated PMNs in comparison to the control.

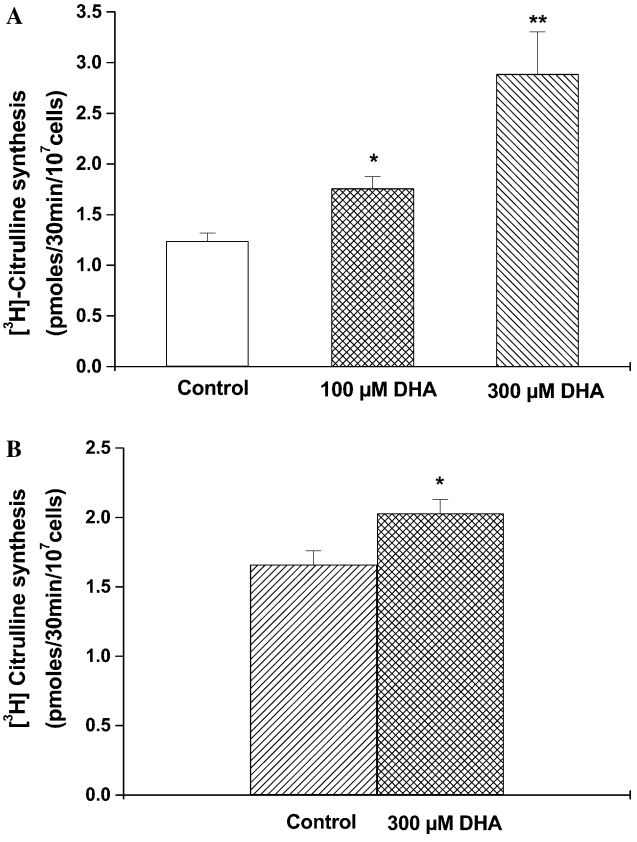

Fig. 2. (A) Bar diagram representing the augmented NOS activity in rat PMNs as indicated by the increased formation of L-[^{3}H]citrulline from L-[^{3}H]arginine in the presence of DHA (100 or 300 μM) compared to control. Values are represented as means ± SEM of five independent experiments in each group. $*P < 0.05$, $**P < 0.01$ on DHA, 100 and 300 μM, respectively, in comparison to control. (B) Histogram representing enhanced NOS activity in monkey PMNs following treatment with 300 μM DHA. Values are represented as means ± SEM of four experiments. $*P < 0.05$ on DHA treatment as compared to control.

Generation of NO in the presence of ascorbate/DHA

A NO specific fluorescent probe, DAF-2DA, was used to monitor the generation of NO from PMNs [16]. NO synthesis was augmented in the presence of ascorbate/DHA in both rat and human PMNs (Fig. 3A). Iodoacetamide, which inhibits intracellular conversion of DHA to ascorbate [17], and subsequently inhibits utilization of DHA, significantly reduced the NO formation (Fig. 3B). NOS inhibition with 7-NI, TFP, or

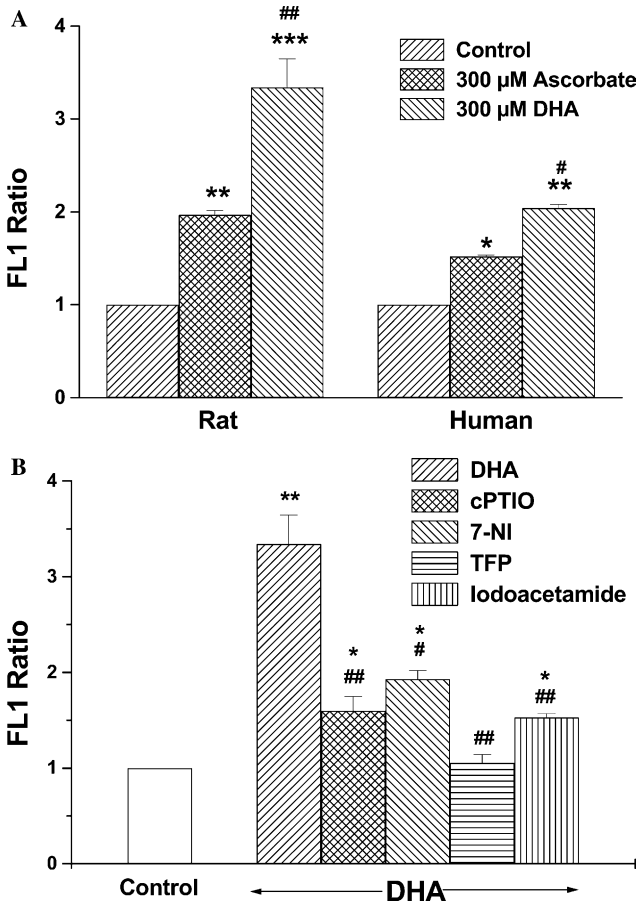

Fig. 3. (A) Generation of NO from the rat PMNs after treatment with ascorbate (300 μM) or DHA (300 μM). NO generation has been represented as the ratio of basal fluorescence (FL1) and fluorescence (See Materials and methods) on treatment with ascorbate or DHA, as measured by a FACS Calibur flow cytometer. Values are represented as means ± SEM of at least 4 experiments in each group. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ in ascorbate or DHA treated PMNs in comparison to control. $\#P < 0.05$, $\#\#P < 0.01$ on DHA treatment in comparison to ascorbate treated PMNs. (B) NO generation, represented by FL1 ratio from control or after treatment with DHA (300 μM) or PMNs pre-treated with a NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO, 30 μM), NOS inhibitors, 7-nitroindazole (7-NI, 1 mM) or trifluoperazine (TFP, 100 μM) or with iodoacetamide (1 mM). $*P < 0.05$, $**P < 0.001$ in DHA treated PMNs in the presence or absence of inhibitors as compared to vehicle (HBSS)-treated PMNs. $\#P < 0.05$, $\#\#P < 0.01$ in the PMNs pre-treated with inhibitors/scavengers and DHA in comparison to only DHA treated PMNs.

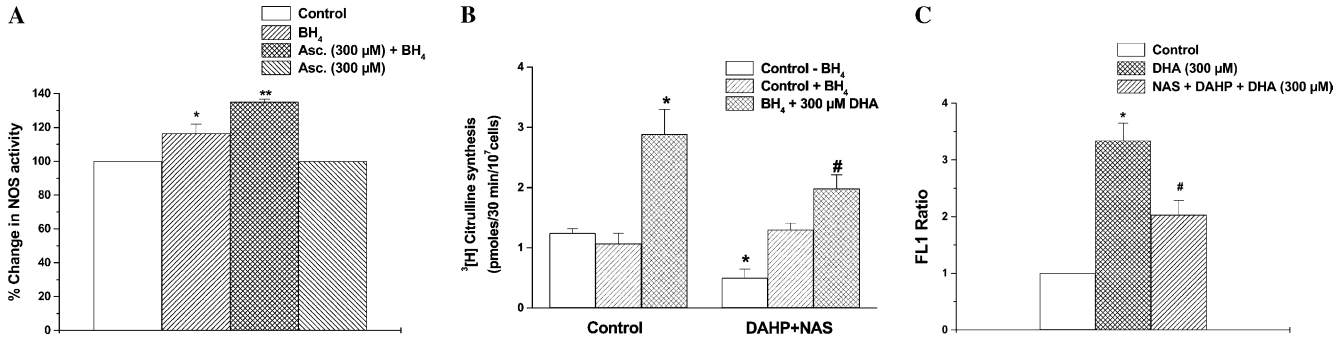

Fig. 4. (A) Bar diagram exhibiting change in the NOS activity in PMN homogenate in the absence or presence of tetrahydrobiopterin (10 μM) and/or the presence of ascorbate (300 μM). Values are represented as means ± SEM of at least 4 independent experiments. *$P < 0.05$, **$P < 0.01$ in comparison to PMN homogenate incubated with cofactors other than BH_4. (B) Bar diagram demonstrating the NOS activity in PMN supernatant pretreated with tetrahydrobiopterin (BH_4) synthesis inhibitors [*N*-acetylserotonin (NAS, 5 mM) and diamino-6-hydroxypyrymidine (DAHP, 5 mM)] in the absence or presence of BH_4 and DHA (300 μM). Values are represented as means ± SEM of at least 4 independent experiments. *$P < 0.05$ in compared to PMNs receiving no BH_4 treatment. #$P < 0.05$ in the BH_4 and DHA treated PMNs in the absence of BH_4 synthesis inhibitors. (C) Histogram representing the attenuation of NO generation, represented by FL1 ratio by flow cytometry in control PMNs treated with DHA (300 μM) in the absence or presence of BH_4 synthesis inhibitors [NAS (5 mM) and DAHP (5 mM)]. Values are represented as means ± SEM of at least 3 independent experiments. * P<0.001 in DHA treated PMNs in comparison to control PMNs. #$P < 0.05$ in the presence of BH_4 synthesis inhibitors [NAS (5 mM) and DAHP (5 mM)] in comparison to only DHA (300 μM) treated PMNs.

NO scavenger cPTIO also inhibited the NO synthesis in DHA treated PMNs.

Modulation of NOS activity in PMNs by tetrahydrobiopterin and ascorbate

In the PMN homogenate, NOS activity was marginally increased after 30 min incubation with tetrahydrobiopterin (10 μM), while ascorbate alone had no effect. Addition of ascorbate (300 μM) prior to tetrahydrobiopterin (10 μM) however significantly enhanced NOS activity (Fig. 4A).

Pretreatment of cells with DAHP (5 mM) and NAS (5 mM) to prevent tetrahydrobiopterin synthesis significantly reduced the NO generation (Fig. 4B), as measured by the attenuated NOS activity. PMNs pretreated with BH_4 synthesis inhibitors exhibited a 32% decrease in the NOS activity following DHA treatment (Fig. 4B).

To ascertain the involvement of BH_4 in NOS activity, PMNs incubated with BH_4 synthesis inhibitors (NAS and DAHP) were loaded with DAF-2DA prior to the addition of DHA (300 μM). NAS and DAHP treatment had no significant effect on the viability or basal fluorescence of resting PMNs (Fig. 4C). The increase in mean fluorescence (FL1) on DHA treatment was significantly reduced in the presence of biopterin inhibitors, confirming the modulatory role of ascorbate in tetrahydrobiopterin to enhance NOS activity and NO generation.

Discussion

Present study investigated the mechanism of ascorbate-mediated increase in NO generation from the isolated PMNs.

Relevance of storing 10–20 mM ascorbate by the PMNs is not well defined. Previous study from our laboratory has demonstrated that pretreatment of PMN suspension with ascorbate modulated platelet aggregability due to enhanced NO release [5]. In the present study, the mechanism of ascorbate and its cell permeable analogue, dehydroascorbate (DHA), on the NO synthesis was analyzed.

PMNs have the ability to uptake both ascorbate and DHA [13], however, it is suggested that PMNs preferentially take up DHA [14,15], and convert it to ascorbate by glutaredoxin, which is inhibited by iodoacetamide [16]. Levels of NO_x were significantly enhanced in the ascorbate/DHA treated PMNs, suggesting an increase in the NO generation by the rat cells. Ascorbate acts as an antioxidant, and, due to its reducing ability, acts also as an oxidant [21–23]. Ascorbate can generate NO by the non-enzymatic reduction of nitrite at low pH. However, we observed that ascorbate enhanced NO generation from PMNs by augmenting NOS activity. Dependence on NOS in nitrite accumulation in rat and monkey PMNs was confirmed by measuring L-[^{3}H]citrulline formation using L-[^{3}H]arginine. The mechanism of ascorbate action was further explored by using the fluorescent dye, DAF-2DA, which rapidly reacts and irreversibly with NO to produce a highly reactive fluorescent product DAF-2T [20]. During the course of this study, it was reported that DAF-2T is not so specific for NO as DAF also reacts with DHA to generate new compounds that have fluorescence emission profiles similar to that of DAF-2T [24], while ascorbate attenuates the fluorescence of DAF-2T. In the present study we measured only the cell-associated fluorescence by flow cytometry and observed that PMNs labeled with DAF-2DA showed increasing

fluorescence on treatment with both ascorbate or DHA (Fig. 3A), suggesting the generation of NO on ascorbate/DHA treatment. Interference with other fluorescent products in the supernatant is unlikely to be quantitated by flow cytometry. Moreover, concentration of ascorbate in the intracellular compartment is higher as compared to that of DHA [17]. We had observed an increase in fluorescence on ascorbate addition also in human PMNs [5]. Involvement of NO in the ascorbate/DHA-mediated response was also confirmed by using a calmodulin antagonist, TFP [25], which in turn inhibits NOS, a NOS inhibitor (7-NI) or a NO scavenger (cPTIO) as suggested by Zhang et al. [24] (Fig. 3B). Our study has therefore convincingly demonstrated the NOS-dependent increase in NO formation by the PMNs after DHA/ascorbate treatment.

Increase in the NO release from PMNs by ascorbate might have beneficial effects on various pathological conditions such as atherosclerosis and diabetes. The results of the present study substantiate the earlier observations that dietary supplementation with vitamin C is beneficial [26–29]. Moreover, chronic granulomatous diseased patients have poor defense against invading microbes due to the defect in ROS generation from PMNs, while an increase in NO production has been found to help combat bacterial infections in these patients [30]. Ascorbate supplementation might thus be more useful in these subjects and this possibility is worth exploring.

PMNs are transcriptionally less active, regulation of NOS activity by ascorbate thus might not be at the transcriptional or translational level. Activity of NOS could also be modulated at the level of cofactor(s) and ascorbate could be interacting with one or more of these cofactors. The enzyme NOS has been subjected to thiol-dependent redox regulation [31,32] and ascorbate could prevent their oxidation and sustain the activity of the enzyme. NOS activity is under the regulation of various cofactors, viz., NADPH, NOS depends on FAD, FMN, tetrahydrobiopterin, and calcium. Recent reports have implicated the key regulation of NOS by tetrahydrobiopterin [33–36].

We observed that addition of ascorbate alone to the PMN homogenate had no effect on NOS activity. However, in the presence of tetrahydrobiopterin, ascorbate significantly enhanced the enzyme activity (Fig. 4A). Moreover, inhibition of tetrahydrobiopterin synthesis by DAHP (5 mM) and NAS (5 mM) reduced the NOS activity as well as NO formation in PMNs (Figs. 4B and C). In these cells, stimulatory effect of DHA was also significantly reduced. Thus, ascorbate seems to interact with tetrahydrobiopterin in the PMNs to augment NO synthesis. Ascorbate in the endothelial cells has also been shown recently to increase the NOS activity by biopterin stabilization [9].

Ascorbate preserves tetrahydrobiopterin by preventing its oxidation [36] or by sparing tetrahydrobiopterin by two mechanisms. First, ascorbate could enhance the affinity of tetrahydrobiopterin for eNOS by preserving crucial thiols required for the cofactor binding [37]. Second, ascorbate could also lessen redox cycling of tetrahydrobiopterin by decreasing intracellular superoxide and peroxynitrite accumulation. Results from our study also seem to implicate that ascorbate plays an important role by preserving tetrahydrobiopterin in NO synthesis by the PMNs. Though role of NO in the PMN bactericidal activity and ROS generation is known [6,7,9], the relative contribution of ascorbate to these activities is relatively less understood and is currently being investigated in our laboratory.

The present study thus demonstrates that ascorbate is indeed utilized by the PMNs to support NO synthesis.

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