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Inhibition of NADPH-Oxidase Activity in Human Polymorphonuclear Neutrophils by Lipophilic Ascorbic Acid Derivatives

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SUMMARY

As recently reported, B-003 (6-*S*-hexadecyl-2-methoxythioascorbic acid) shows strong inhibition of the *N*-formylmethionylleucyl phenylalanine (fMLP)-stimulated neutrophil superoxide production and degranulation *ex vivo*, which is not correlated with its antioxidant properties. Structure-activity studies with 12 derivatives, together with permeation studies, pointed to a process for uptake of B-003 but not its regioisomer B-015 into neutrophils and revealed the importance of the free acidic enolic hydroxyl group in the 3-position of ascorbic acid and of a long chain alkyl group having a chain length of C_{16}-C_{18} for effective inhibition. We now report that B-003 also strongly suppressed C5a-, concanavalin A-, and calcium ionophore A23187-stimulated superoxide formation, whereas protein kinase C-mediated activation by phorbol ester remained unaffected. The fMLP- or C5a-induced calcium mobilization from intracellular stores of fura-2-loaded cells, as well as the fMLP- or A23187-triggered release of [^{14}C] arachidonate from prelabeled neutrophils, was not affected by B-003. The observed release of GSH was not causally related to inhibition of the oxidative burst, because GSH depletion by 1-chloro-2,4-dinitrobenzene was without effect on the fMLP-stimulated superoxide formation or on the inhibitory effect of B-003. In a cell-free system, consisting of a light membrane fraction and a cytosol fraction from resting neutrophils, B-003 inhibited the arachidonate-induced assembly of the NADPH-oxidase under conditions where particulate NADPH-oxidase from phorbol ester-preactivated neutrophils and catalytically active cell-free assembled oxidase were not affected. The inhibitory effect was more pronounced when the system was incubated in the presence of the G protein activator guanosine-5′-*O*-(3-thio)triphosphate (GTPγS). [^{35}S]GTPγS binding studies excluded displacement of the G protein activator from guanine nucleotide binding sites by B-003. *In vitro* assembly/co-sedimentation experiments in the presence of GTPγS revealed a 2-fold increase in a small cytosolic G protein with a molecular mass of 21 kDa (p21) in pelleted membranes, as detected by [^{35}S]GTPγS protein blot probing, that was not affected by B-003. Structure-activity relationship studies of the effects of various 6-*S*-alkylascorbyl derivatives on the GTPγS/arachidonate-triggered assembly of the NADPH-oxidase showed strong dependence of the inhibition on the alkyl chain length, with long chain alkyl derivatives (C_{16} and C_{18}) being most effective. Our results suggest that the 6-*S*-hexadecylascorbyl compound B-003 inhibits the oxidative burst by interfering with the Ca^{2+}-dependent but protein kinase C-independent assembly of the NADPH-oxidase.

Inhibition of neutrophil functions, especially the chemotactic response, oxygen radical production, and granule secretion, would be of prime importance for treating inflammations. Therefore, starting with our initial observation that ascorbyl-6-palmitoate could block leukotriene B_4 synthesis, the oxidative burst, and secretion in human neutrophils (1), it seemed worthwhile to synthesize new and more potent lipophilic derivatives of ascorbic acid. This was encouraged by recent reports on 2-*O*-octadecyl ascorbic acid (CV-3611) as an effective agent against postischemic damage, which seemed to correlate well with its antioxidative activity (2, 3). Within a series of twelve 6-thio-ascorbate derivatives, we found that B-003 (6-*S*-*n*-hexadecyl-2-*O*-methyl-6-thio-L-ascorbic acid) potently inhibited superoxide generation and also granule secretion (4). B-003 also possessed antioxidant properties in a lipid peroxidation assay, but no correlation of the antioxidant potential with inhibition of the oxidative burst or release of granule contents could be established. Moreover, it was determined that B-003 but not its regioisomer B-015 (6-*S*-*n*-hexadecyl-3-*O*-methyl-6-

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ABBREVIATIONS: PMN, polymorphonuclear neutrophils; fMLP, *N*-formylmethionylleucyl phenylalanine; Con A, concanavalin A; IP_3, inositol-1,4,5-trisphosphate; PMA, phorbol-12-myristate-13-acetate; PLA_2, phospholipase A_2; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide; CDNB, 1-chloro-2,4-dinitrobenzene; SOD, superoxide dismutase; DFP, diisopropyl fluorophosphate; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; GDPβS, guanosine-5′-*O*-(2-thio)diphosphate; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; AA, arachidonic acid; *phox*, phagocyte oxidase; PBSS, supplemented phosphate-buffered saline; PKC, protein kinase C.

thio-L-ascorbic acid) accumulated in PMN and that part of the action of B-003 could be attributed to the higher effective concentration in PMN. The decrease in intracellular pH observed with B-003 but not with B-015 could be excluded as a determinant in the inhibition of the oxidative burst. Because CV-3611 exhibited properties very similar to those of B-003 but for both compounds the inhibitory mode of action had not yet been elucidated, we studied the effects of this class of compounds on presumed parameters of neutrophil signal transduction, such as intracellular Ca^{2+} mobilization and AA release, in more detail. In addition, to eliminate the observed effects of different levels of accumulation of the lipophilic ascorbic acid derivatives seen in intact cells, we expanded our structure-activity studies in cell-free NADPH-oxidase preparations.

Because our previous results were obtained with fMLP as an agonist for PMN, we examined the effects of B-003 on the oxidative burst triggered by other known receptor-mediated activators, such as complement factor C5a and the plant lectin Con A, as well as receptor-independent stimuli such as the calcium ionophore A23187 and phorbol esters.

After receptor binding, fMLP and C5a are thought to trigger phosphatidylinositol-4,5-bisphosphate hydrolysis by activation of a phosphoinositide-specific phospholipase C, via a membranous heterotrimeric G protein (G_c). The phosphoinositide response results in diacylglycerol and IP_3 formation, and IP_3 triggers a subsequent increase in intracellular Ca^{2+} (5–10). The Ca^{2+} ionophore A23187 alone can also initiate the oxidative burst without hydrolysis of phosphatidylinositol-4,5-bisphosphate and, similarly to Con A, increases intracellular Ca^{2+} in a G_c-independent manner (11–13). How the Ca^{2+} increase triggers the onset of the oxidative burst is not well understood. Ca^{2+}-dependent activation of PLA_2 and subsequent release of AA is discussed by some authors (14, 15) and supported by the blocking effects of PLA_2 inhibitors on superoxide formation (16–19). Activation of the NADPH-oxidase by low concentrations of AA has been reported for electropermeabilized PMN (20), and in several reconstituted cell-free systems AA has been shown to initiate the assembly of the NADPH-oxidase (21, 22).

Human neutrophil NADPH-oxidase consists of a membranous heterodimeric *b*-type cytochrome (containing p22-*phox* and p91-*phox*) and at least two cytosolic components (p47-*phox* and p67-*phox*), which after cellular or cell-free activation assemble to the active O_2^--generating enzyme complex (23–25). p47-*phox* and p67-*phox*, which have been cloned and sequenced (26, 27), translocate to the cellular membrane after cellular and cell-free activation (28–30). Although the exact mechanism responsible for this translocation is not known, Lomax *et al.* (26) speculate that this could be due to the observed intense phosphorylation of a series of basic amino acids next to the amino terminus of p47-*phox*.

There are several recent reports pointing to a role of nonheterotrimeric G proteins in the activation and/or regulation of the NADPH-oxidase (31–35). In an AA-activated cell-free oxidase system, consisting of membranous and cytosolic fractions of resting neutrophils, guanine nucleotides like GTPγS enhance the enzyme activity severalfold (31, 35), and immunodepletion of cytosol from resting neutrophils by an anti-*ras* antibody leads to pronounced inhibition of cell-free NADPH-oxidase activity (34). Quinn *et al.* (32) reported a direct association of a membranous low molecular weight G protein, rap1a/K*rev*1, with the *b*-type cytochrome, although its function is not clear. Recently, cytosolic guanine nucleotide-dependent proteins with a molecular mass of about 21 kDa were purified from macrophages as well as neutrophils and were shown to be members of the superfamily of ras proteins with sequence homology to rac1 (33) and rac2 (36). Both low molecular weight G proteins were able to augment the rate of O_2^- production in a reconstituted cell-free oxidase activation system, and rac1 has been shown to be associated with a ras regulatory protein (37, 38). The physiological relevance of these *in vitro* findings and the role of this nonheterotrimeric G protein in neutrophil signal transduction, however, have not yet been clarified.

Results in the literature clearly indicate that a second pathway for NADPH-oxidase activation exists through PKC (14, 17, 39–41). PMA and other phorbol esters can also mediate the oxidative burst, which seems to be due to phosphorylation and subsequent translocation of the p47-*phox* subunit of the NADPH-oxidase (28, 42). PKC inhibitors inhibit this reaction but show no effects or minor inhibitory effects on the fMLP- or C5a-activated burst, indicating that this PKC-dependent pathway is of minor importance under more physiological activation conditions (39). The existence of a second pathway allowed, however, discrimination of the action of the lipophilic ascorbic acid derivatives, because we provide evidence that these compounds affect only the Ca^{2+}-dependent signal transduction pathway in human PMN.

Experimental Procedures

Materials

B-003, B-015, and all other 6-*S*-alkylascorbyl derivatives were synthesized as described previously (4). Ficoll-Paque, cytochrome *c* (type III), A23187, PMA, fMLP, Con A, *p*-bromophenacyl bromide, CDNB, EGTA, EDTA, DFP, Tween 20, sucrose, sulfosalicylic acid, and sodium azide were obtained from Sigma Chemical Co. (Deisenhofen, Germany). SDS and acrylamide/bisacrylamide were purchased from Bio-Rad. GTPγS, GDPβS, ATP, dithiothreitol, dithioerythritol, NADPH, HEPES, luminol, and leupeptin were obtained from Boehringer-Mannheim (Mannheim, Germany). AA was purchased from Larodan (Malmö, Sweden). Fura-2/acetoxymethyl ester and monobromobimane (Thiolyte) were obtained from Calbiochem (Frankfurt, Germany). [1-^{14}C]AA and [^{35}S]GTPγS were from New England Nuclear (Dreieich, Germany). All other reagents were commercially available and of at least reagent grade.

Methods

Preparation of neutrophils. The preparation of human neutrophils was performed by dextran sedimentation, subsequent centrifugation on Ficoll-Paque, and hypotonic lysis of contaminating erythrocytes, as described previously (1). The purity of the PMN was about 95% and the viability measured by trypan blue exclusion was >97%. After isolation the cells were stored at 4° at a cell density of 2×10^7/ml in PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 280 mOsM).

Measurement of superoxide radical production by cytochrome *c* reduction. Superoxide radical production of activated neutrophils was measured as SOD-sensitive cytochrome *c* reduction as described previously (4), with slight modifications. In brief, after a 3-min preincubation in PBSS (1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose) at 37°, PMN (2×10^6/ml) were stimulated as indicated, in the presence of 80 μM cytochrome *c*.

For measurement of the cell-free NADPH-oxidase activity, 3–5 μg of light membrane fraction and 35–50 μg of cytosol in incubation buffer (250 μl) were incubated in the presence or absence of 10 μM GTPγS for 2 min. After initiation of the assembly with 50 μM AA and 3-min incubation at 27°, the assembly mixture was transferred to a cuvette

containing 250 μl of incubation buffer plus cytochrome *c* (50 μM final concentration). After base-line monitoring for 1–2 min, enzyme activity was initiated with 200 μM NADPH. Superoxide radical formation was continuously detected at 550 nm/538 nm with a dual-wavelength spectrophotometer (Aminco DW2). Cytochrome *c* reduction was confirmed as NADPH-oxidase activity by inhibition with 50 units/ml SOD. For calculations, an extinction coefficient for cytochrome *c* of 21.5 mM^{-1} cm^{-1} at 550 nm was used.

Luminol-enhanced chemiluminescence. PMN were preincubated for 5 min at 37° in PBSS containing 5 μM luminol. After an additional 3-min incubation with the indicated substances (0.1% DMSO, final concentration), cells were activated by various stimuli and chemiluminescence was continuously monitored with a chemiluminometer (model 1240; LKB-Wallac) connected to a chromatointegrator (model D2000; Merck-Hitachi). Peak areas were used for calculation of the percentage inhibition values.

Measurement of NADPH:O_2 oxidoreductase activity. NADPH:O_2 oxidoreductase (NADPH-oxidase) activity was measured (with a Clark oxygen electrode) as the rate of oxygen consumption at 27°, under constant stirring. The assay mixture (0.5 ml) was composed of incubation buffer (5% sucrose, 10 mM HEPES, 10 mM potassium phosphate, 70 mM NaCl, 0.5 mM EGTA, 1.5 mM $MgCl_2$, 1 mM sodium azide, pH 7.0), 10 μg of the light membrane fraction, and 200 μg of cytosol. Where indicated in the text, 10 μM GTPγS was added 2 min before activation with 80 μM AA. After assembly of the oxidase for 3 min and base-line monitoring, enzyme activity was triggered with 200 μM NADPH. For calculation of the NADPH-oxidase activity, 286 nmol of O_2/ml of incubation buffer was used as 100% saturation.

Intracellular calcium mobilization. Neutrophil cytosolic calcium mobilization was determined by the fura-2 method, as described by Grynkiewicz *et al.* (43). Fura-2-labeled cells (2×10^6/ml) in PBSS were preincubated with 2.5 μM B-003 (0.1% DMSO) at 37° for 3 min, with continuous stirring, and the cells were stimulated with the indicated concentrations of fMLP or C5a. Fura-2 fluorescence was monitored continuously at excitation wavelengths of 335 nm and 362 nm, using a dual-excitation fluorescence spectrophotometer (Sigma ZSW II; Fa. Biochem, Puchheim, Germany).

Measurement of AA liberation. Neutrophils (1×10^7/ml) were labeled with 1–2 μM [1-^{14}C]AA (60 mCi/mmol) for 15 min at 37° in Ca^{2+}-free PBS (1 mM $MgCl_2$, 5.5 mM glucose). After centrifugation, cells were resuspended in Ca^{2+}-free PBS and incubated again with 1 μM [1-^{14}C]AA for 15 min at 37°. After this, the cells were washed twice with prewarmed PBS (1 mM $MgCl_2$, 5.5 mM glucose) containing 1 mg/ml essentially fatty acid-free bovine serum albumin. Of the exogenously added AA, 80–90% was incorporated by this procedure. Finally, the cells were resuspended in Ca^{2+}-free PBS until used for incubations.

[^{14}C]AA-labeled PMN (5×10^6/ml) were preincubated for 10 min at 37° in PBSS. After addition of the indicated substances and further incubation for 3 min, cells were stimulated with 100 nM fMLP or 2 μM A23187. Fifteen seconds after stimulation, fatty acid-free bovine serum albumin (0.4% final concentration) was added and the cells were incubated for an additional 15 min. The cells were then centrifuged in an Eppendorf microfuge at full speed for 1 min and placed on ice. Duplicate 600-μl aliquots of the supernatant were counted by liquid scintillation counting.

Determination of glutathione concentrations. Intracellular GSH levels of human PMN were measured with the monobromobimane method, using a coupled high performance liquid chromatography/spectrofluorimetric analytical system, as described previously (44). In brief, PMN (5×10^6/ml) were preincubated in supplemented PBSS at 37° with the indicated substances. After 5 min the reaction was stopped with 200 μl of ice-cold formic acid (1%) and the cells were pelleted by a short centrifugation. The pellet was resuspended in 80 μl of 5% sulfosalicylic acid containing 50 μM dithioerythritol and was precipitated for 30 min on ice. After neutralization of nonprecipitated material with 1 M *N*-ethylmorpholine and addition of monobromobimane, the $HClO_4$-stopped aliquots were assayed for GSH as described.

Isolation of light membrane fraction and cytosol from resting neutrophils. Neutrophils were disrupted and fractionated by the procedure described by Bolscher *et al.* (35), with slight modifications. In brief, after pretreatment with 1 mM DFP for 10 min at 4°, neutrophils were resuspended in ice-cold lysis buffer (5% sucrose, 10 mM HEPES, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 100 μM leupeptin, pH 7.0) at a cell density of 1×10^8/ml. After sonication (3×15 sec, 30% output) of the neutrophil suspension and removal of unbroken cells and nuclei, 0.9 ml of the postnuclear supernatant was layered on a discontinuous sucrose gradient consisting of 1.5 ml of 40% (w/v) sucrose and 1.5 ml of 15% (w/v) sucrose. After ultracentrifugation (100,000 × *g*, 45 min), light membranes were collected from the interface of the sucrose layers, whereas cytosol was collected to the same volume (0.9 ml) from the top. Cytosol from the top layers was pooled and recentrifuged at 200,000 × *g* for an additional 30 min, in a fixed-angle rotor (Beckman TL100 ultracentrifuge), to remove residual membranes. Cytosol and particulate protein concentrations were determined with the Pierce bicinchoninic acid protein assay kit. The resulting supernatants and light membranes were stored at −80°.

Particulate NADPH-oxidase was prepared from PMA-preactivated neutrophils. In brief, after 10 min of preincubation with 1 mM DFP, PMN were resuspended in PBSS at a cell density of 5×10^6/ml and stimulated with 100 nM PMA for 10 min. After pelleting, the cells were adjusted to 1×10^8/ml in ice-cold lysis buffer and fractionated as described above. Light membranes were collected from the sucrose interface and used for particulate oxidase measurements.

***In vitro* assembly/co-sedimentation experiment.** *In vitro* assembly of the NADPH-oxidase was done as described above. In brief, after a 3-min assembly of light membranes (25 μg) and cytosol (500 μg) in 1.25 ml of incubation buffer with 80 μM AA, in the presence or absence of 10 μM GTPγS, the assembly mixture was cooled on ice for 5 min. After addition of 10 mM $MgCl_2$, the reaction mixture was centrifuged immediately at 4° and 200,000 × *g* for 5 min, using a Beckman TL100 ultracentrifuge. The supernatant was removed and the pellet was carefully resuspended in 60 μl of incubation buffer. Aliquots were used for SDS-PAGE/protein blot probing (40 μl) and for measurement of NADPH-oxidase activity (10 μl). For oxidase activity measurements, the aliquot was diluted in 500 μl of incubation buffer containing 50 μM cytochrome *c*, and enzyme activity was initiated with 200 μM NADPH. Superoxide radical formation was continuously monitored by SOD-sensitive cytochrome *c* reduction, as described above.

[^{35}S]GTPγS binding. The influence of B-003 on the binding of radiolabeled GTPγS to G proteins was determined by a slightly modified filtration technique, as described previously (45). In brief, light membranes (2.5 μg), cytosol (50 μg), or complete assembly mixtures (consisting of 2.5 μg of light membranes, 50 μg of cytosol, and 50 μM AA in incubation buffer) were incubated for 5 min at 27° with 10 μM [^{35}S]GTPγS (1262 Ci/mmol). The reaction was stopped by transfer to 2 ml of ice-cold filtration buffer (20 mM Tris·HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$), followed by rapid filtration on nitrocellulose filters (BA-85, 0.45 μm; Schleicher & Schüll). Filters were washed three times with the same ice-cold buffer. After the filters were dissolved in 10 ml of scintillation mixture, bound radioactivity was counted by liquid scintillation counting. Nonspecific binding to denatured membranous or cytosolic protein was approximately 20% and was subtracted from the resulting counts.

SDS-PAGE and protein blotting. Samples, suspended in electrophoresis sample buffer (10 mM sodium phosphate, 2%, w/v, SDS, 5%, v/v, β-mercaptoethanol, 10%, v/v, glycerol, 0.01%, v/v, pyronine, pH 6.8), were separated by SDS-PAGE using a 12% mini-slab gel, according to the method of Laemmli (46). Proteins were transferred to nitrocellulose for 50 min at 200 mA under wet blotting conditions, using blotting buffer (25 mM Tris·HCl, 150 mM glycine, 10% methanol, pH 8.5). Transfer of sample and marker proteins was controlled by reversible Poinceau staining after each run.

[^{35}S]GTPγS protein blot probing. For the detection of low molecular weight G proteins, nitrocellulose sheets were labeled with [^{35}S]

GTPγS by a slight modification of the procedure described by Bokoch and Parkos (47) and Drobak *et al.* (48). In brief, transfer sheets were immersed in 20 ml of 50 mM Tris·HCl, pH 7.5, 0.3% (v/v) Tween 20, 0.5 mM EDTA, 0.55 mM $MgCl_2$, 100 μM dithiothreitol, and were incubated for 30 min at room temperature. The solution was decanted and the nitrocellulose sheet was incubated for an additional 30 min in 10 ml of the same buffer containing 1 μM [^{35}S]GTPγS (1262 Ci/mmol). Nonspecific binding sites were blocked by addition of 10 μM ATP 1 min before addition of radiolabeled GTPγS. The blots were washed three times with ice-cold Tris·HCl, pH 8.0, containing 5 mM $MgCl_2$ and, after thorough drying, were exposed to a PhosphoImager screen for 15–16 hr.

Results

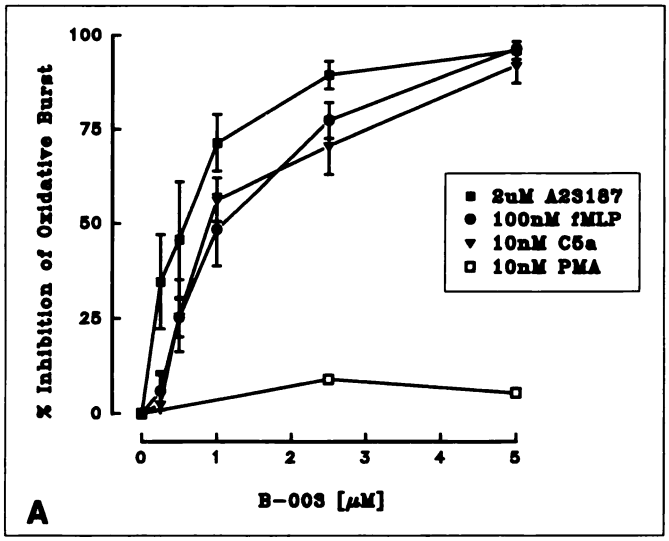
Previous experiments on the inhibition of the oxidative burst in human PMN by lipophilic ascorbic acid derivatives were carried out with fMLP as a stimulus. To exclude a specific interaction with the fMLP receptor, PMN were preincubated with the compound B-003 and then stimulated with 10 nM C5a, 2 μM A23187, or 10 nM PMA (Fig. 1A). The oxidative burst triggered by the first two agents was blocked efficiently, with IC_{50} values similar to those obtained with 100 nM fMLP. Surprisingly, no effect was seen with 10 nM PMA. Because PMA caused a much stronger activation of the burst than did fMLP or C5a, lower concentrations of PMA (0.5, 1.0, and 5.0 nM) were also tested, and again no inhibition but rather a slight activation was observed (data not shown). B-003 was effective also during the ongoing burst, as shown in Fig. 1B, *trace c*. Conversely, the fMLP-triggered and B-003 (5 μM)-inhibited oxidative burst could be evoked by 10 nM PMA, confirming the postulated two separate pathways for NADPH-oxidase activation (16, 39, 41).

According to these results, B-003 seemed to interfere with the signal transduction pathway leading to the liberation of intracellular Ca^{2+} through the phosphoinositide response. However, when the release of Ca^{2+} was monitored by fura-2 fluorescence, no change in the Ca^{2+} signals occurred (Fig. 2). Obviously, none of the events after receptor activation by C5a or fMLP and before the release of intracellular Ca^{2+} was affected by B-003.

Con A differs in its signaling pathway from C5a and fMLP, by stimulating the oxidative burst in a Ca^{2+}-dependent but pertussis toxin- and IP_3-insensitive manner (7, 12, 13). With 100 μg/ml Con A, a concentration used by other investigators, no significant difference in the inhibition, compared with C5a and fMLP, was seen. Due to the weak superoxide release triggered by Con A, this test was performed with the more sensitive chemiluminescence assay. Under those assay conditions we observed the same lack of effect of B-003 with PMA as a stimulant (Fig. 3).

Assuming that Ca^{2+} was required for the activation of the NADPH-oxidase, we measured the release of [^{14}C]AA from prelabeled PMN. No influence of B-003 on the modest increase after fMLP was seen, with a slight but barely significant inhibition after A23187 treatment (Fig. 4). As a control we incubated the rather nonspecific PLA_2 inhibitor *p*-bromophenacyl bromide (10 μM) with PMN and then stimulated cells with fMLP. The AA levels decreased to basal, and the oxidative burst was inhibited to about 80% (data not shown). Thus, if AA indeed plays a role in the activation of the burst, B-003 would not interfere with its liberation.

As described previously, B-003 caused an efflux of reduced glutathione from PMN, and this could have been causally connected to a loss of NADPH-oxidase activity. To test this possibility, we depleted PMN of GSH by incubation with the GSH-*S*-transferase substrate CDNB and then stimulated cells with fMLP (Fig. 5). The oxidative burst proceeded unaffected but again was blocked by subsequent addition of B-003. The possibility that a lack of GSH was the cause of the observed



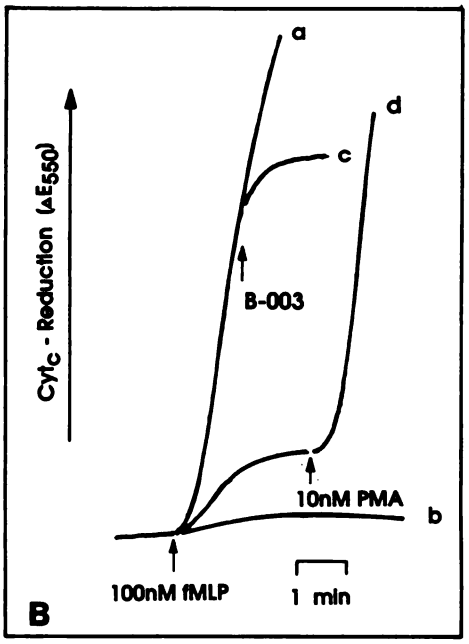


Fig. 1. Effect of B-003 on the oxidative burst of human PMN after activation with different stimuli. A, Concentration-dependent inhibition. Cells (2×10^6/ml, in PBSS) were preincubated for 3 min with the indicated concentrations of B-003 (0.1% DMSO) and stimulated with 100 nM fMLP (●), 10 nM C5a (▼), 2 μM A23187 (■), or 10 nM PMA (□). Superoxide radical generation was detected by SOD-sensitive cytochrome *c* reduction and continuously monitored with a dual-wavelength spectrophotometer (537 nm/550 nm). Values are given as means ± standard errors from three to five independent experiments. B, Reversibility of the inhibitory effect of B-003. *Trace a*, fMLP control; *trace b*, inhibition of the fMLP-triggered burst after 3-min preincubation with 5 μM B-003; *trace c*, application of 5 μM B-003 (*arrow*) after ongoing burst; *trace d*, restoration by PMA of the B-003 (2.5 μM)-inhibited fMLP-triggered burst. Representative tracings of three independent experiments are shown.

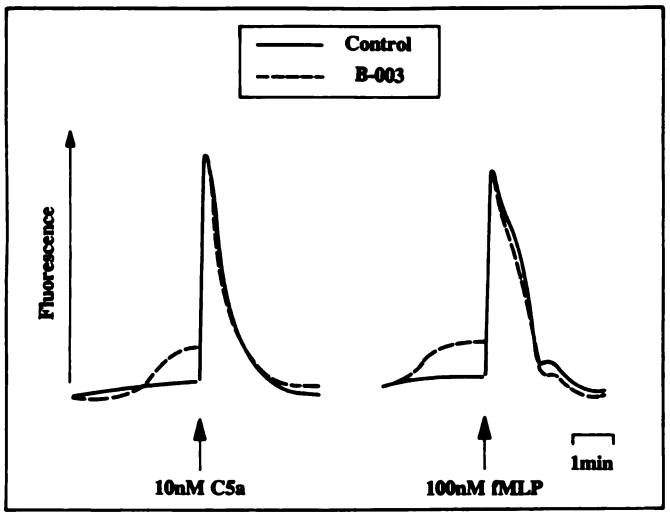


Fig. 2. Influence of B-003 on the intracellular calcium mobilization of human PMN. Fura-2-loaded cells (2×10^6/ml, in PBSS) were equilibrated at 37° for 5 min. After addition of 2.5 μM B-003 and an additional 3-min incubation, cells were stimulated with 100 nM fMLP or 10 nM C5a. Fura-2 fluorescence was continuously monitored as described in Experimental Procedures. Representative tracings of three independent experiments with cells of different donors are shown.

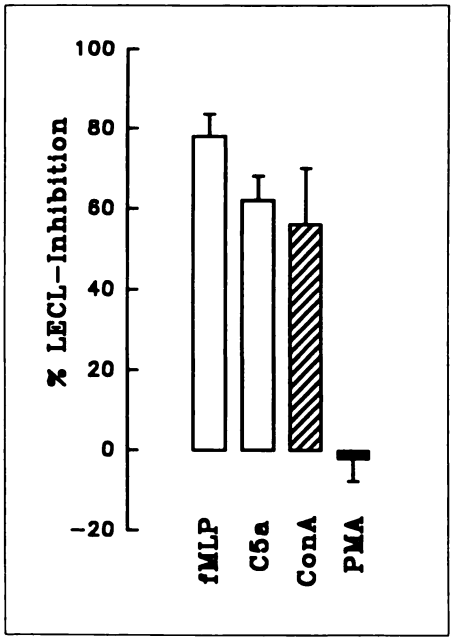


Fig. 3. Inhibition of the oxidative burst of human PMN by B-003, measured by luminol-enhanced chemiluminescence (*LECL*). PMN (2×10^6/ml) were preincubated in PBSS (with 5 μM luminol) with 2.5 μM B-003 (0.1% DMSO) for 3 min before stimulation with the indicated stimuli. Luminol-enhanced chemiluminescence was continuously detected with a chemiluminometer and analyzed as described in Experimental Procedures. Data are given as means ± standard errors from three to five independent experiments.

inhibition of the oxidative burst by B-003 could therefore be excluded.

Before examining possible effects of the inhibitory ascorbic acid derivative on more recently discovered additional signaling pathways (49, 50), we investigated a possible disturbance of the assembly of the components of the NADPH-oxidase complex by B-003. Because cell-free preparations have recently become

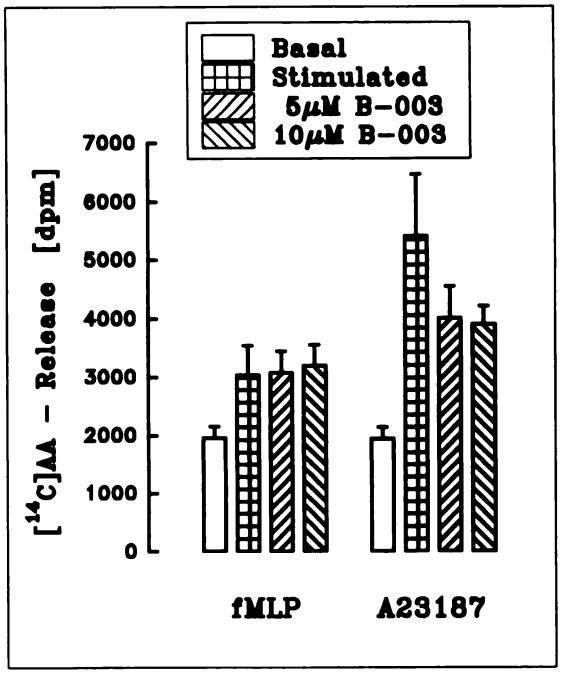


Fig. 4. Influence of B-003 on the fMLP- and A23187-stimulated release of [1-^{14}C]AA from human PMN. [1-^{14}C]AA-labeled cells (5×10^6/ml) were equilibrated at 37° for 5 min in supplemented HEPES buffer (with 5 mM Ca^{2+}, 1 mM Mg^{2+}, and 5.5 mM glucose). After 3-min preincubation with 5 μM or 10 μM B-003, cells were stimulated with 100 nM fMLP or 2 μM A23187 and the radioactivity in the supernatant was detected as described in Experimental Procedures. Values are given as means ± standard errors from four independent experiments.

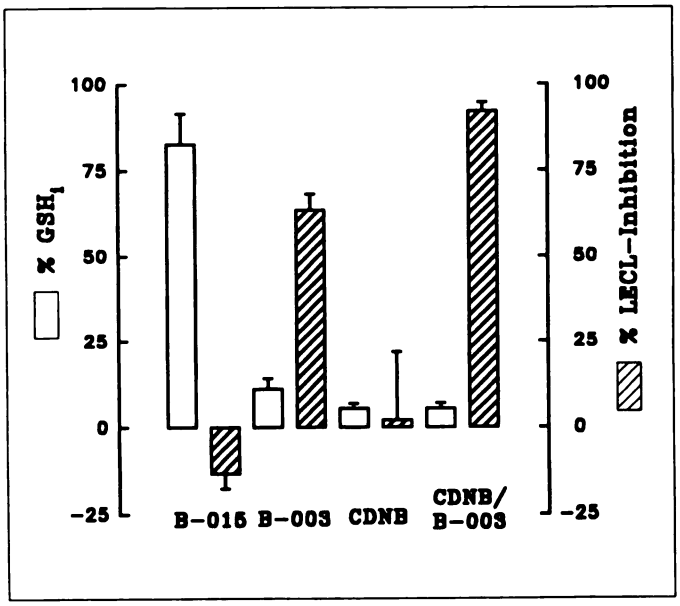


Fig. 5. Effect of CDNB on the fMLP-stimulated burst and intracellular level of GSH, in comparison with B-003. PMN (5×10^6/ml) were preincubated with 100 μM CDNB, 5 μM B-015, or 5 μM B-003 for 5 min at 37°. In the two rightmost columns, cells were preincubated with 100 μM CDNB for 5 min and, after addition of 5 μM B-003 and additional incubation for 2 min, stimulated with 100 nM fMLP. After preincubation, cells were either stimulated with fMLP and assayed for superoxide formation by luminol-enhanced chemiluminescence (*LECL*) or assayed for changes in intracellular GSH as described in Experimental Procedures. Values are given as means ± standard errors from three or four independent experiments.

available, this hypothesis could be tested experimentally. For such studies it had to be considered that B-003 accumulated in PMN, so that cell-free preparations had to be tested in a higher concentration range.

We first selected a preparation obtained after PMA stimulation of intact neutrophils. This treatment was shown to cause phosphorylation of the p47-*phox* subunit, probably by activation of PKC (51, 52). This assembly resists even sonification, and hence active membrane fractions producing superoxide in the presence of NADPH and oxygen can be isolated.

Pretreatment of active membrane fractions (particulate NADPH-oxidase) with 40 μM B-003 did not diminish the activity of such membrane fractions, as shown by the four experiments in Table 1. Similarly, preincubation of PMN (5×10^6/ml) with 5 μM B-003 before stimulation with PMA and subsequent preparation did not affect the activity of particulate NADPH-oxidase (data not shown). This confirmed the *ex vivo* experiments of Figs. 1 and 3.

The second pathway of NADPH-oxidase activation also involves the association of p47-*phox* and p67-*phox* with the membranous cytochrome *b* subunit, but instead of PKC a low molecular weight G protein seems to be involved, because a reconstituted system from membranes and cytosol requires GTPγS and is blocked by GDPβS. In addition, higher concentrations of amphiphiles such as AA or SDS are necessary in this system. When such a system was reconstituted with 80 μM AA and GTPγS, we observed an inhibition of oxygen consumption after preincubation with B-003, as well as with the regioisomer B-015, which was found to be inactive in whole PMN. Both compounds were required at 30 μM to decrease the enzyme activity to almost basal levels (Fig. 6A). Preincubation of the system with 40 μM B-003 or B-015 resulted in much stronger inhibition, with activities being 20–30% of the basal values (data not shown). When B-003 was added to the catalytically active enzyme complex after AA and NADPH, the activity could not be blocked (Fig. 6B).

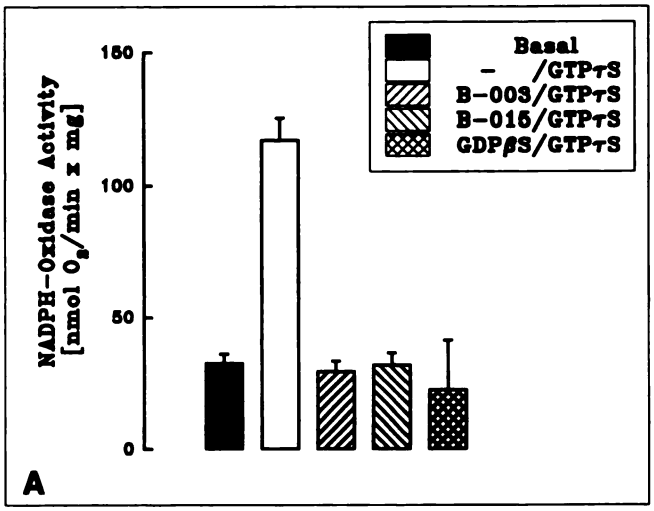
When 500 μM GDPβS was used, we observed an inhibition of the oxidase activity down to basal levels similar to that seen with both ascorbic acid derivatives. This, however, required a prolonged preincubation time of 10 min and a low-Mg^{2+} buffer before Mg^{2+}/GTPβS addition. This observation was in agreement with recent findings describing the guanine nucleotide exchange of the purified ras-like G protein rac2 being faster, but still in the range of minutes, at low Mg^{2+} concentrations (53). At variance to this, the inhibition by B-003 and B-015

TABLE 1

Influence of B-003 and B-015 on particulate NADPH-oxidase activity

Catalytically active NADPH-oxidase was prepared from PMA-preactivated neutrophils as described in Experimental Procedures. In brief, light membranes (20 μg) were preincubated with 40 μM B-003 or 40 μM B-015 (0.2% DMSO) for 3 min at 27°. Oxidase activity was triggered with 200 μM NADPH, and the oxygen consumption was continuously monitored with a Clark-type electrode. Values from experiments with membrane fractions from three to five preparations are shown.

Expt.	Specific activity		
	Control	40 μM B-003	40 μM B-015
	nmol/min/mg		
1	131.3	141.4	
2	248.5	213.3	
3	163.4	138.0	158.9
4	400.0	389.9	341.5
5	222.0	223.8	229.3
Mean ± SD	233 ± 104.2	221.3 ± 102.2	243.2 ± 92.1



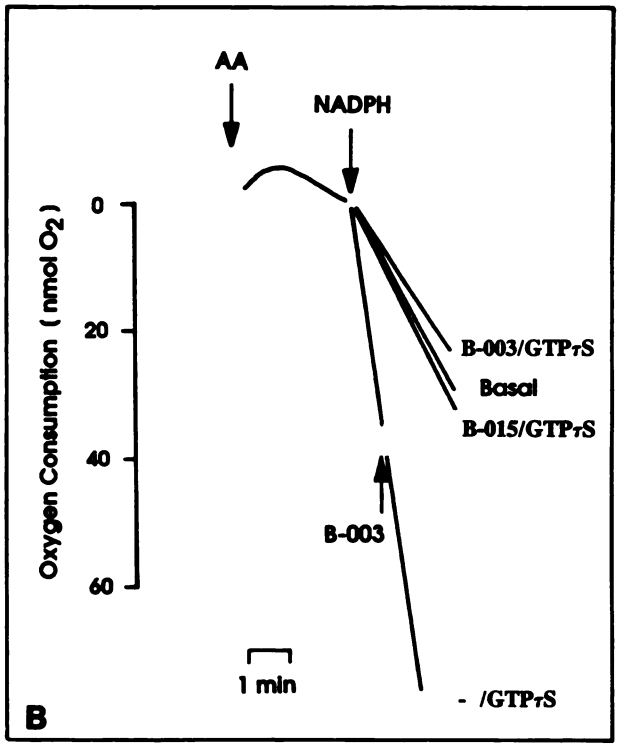


Fig. 6. Inhibition of the cell-free GTPγS/AA-induced NADPH-oxidase activation by B-003 and B-015. Cytosolic and membrane fractions were prepared and incubated in the cell-free NADPH-oxidase system as described in Experimental Procedures. After 3-min preincubation with 30 μM B-003 or 30 μM B-015 (0.2% DMSO) at 27°, the oxidase assembly was triggered with 80 μM AA in the presence or absence of 10 μM GTPγS, as indicated. After 3 min of additional incubation, NADPH-oxidase was activated by 200 μM NADPH and the enzyme activity was monitored polarographically for 3–5 min. A, Summary of the results. GDPβS (500 μM) was preincubated for 10 min in Mg^{2+}-deficient incubation buffer, followed by addition of 5 mM $MgCl_2$ and 10 μM GTPγS. Assembly and activity measurement conditions were identical to the standard assay conditions described above. Results are means ± standard errors of four to eight experiments with membranous and cytosolic fractions from different donors. B, Representative tracings. Where indicated (*arrow*), 30 μM B-003 was added to the assembly mixture.

was seen after a 1-min preincubation and at high Mg^{2+} concentrations.

Because of the apparent amphiphilic properties of B-003 and B-015 and due to the requirement for detergents in the cell-free system, we compared the effects of other anionic amphiphiles, such as SDS (30 μM), deoxycholate (30 μM), and saponin (10 μg/ml), on the reconstituted system, but only with the latter detergent could a 5–40% inhibition be observed (data not shown).

Due to the pronounced inhibitory effect of B-003 and B-015 on the GTPγS-enhanced activity of the cell-free assembled NADPH-oxidase and due to the presumed involvement of the cytosolic low molecular weight G protein rac2 in the reconstituted oxidase system, we studied the influence of the ascorbic acid derivatives on the binding of radiolabeled GTPγS. When 10 μg of light membranes or 100 μg of cytosol were incubated in the presence of 20 μM B-003 or 20 μM B-015, as shown in Fig. 7, the binding of [^{35}S]GTPγS to either fraction was not affected by the compounds. To obtain a more direct comparison with the inhibitory effects seen on the AA-stimulated assembly of the NADPH-oxidase, we examined the influence of B-003 and B-015 on the binding of [^{35}S]GTPγS under assembly conditions. As can be see in Fig. 7, neither of the compounds significantly affected the guanine nucleotide binding. Nonspecific binding of GTPγS was controlled by heat treatment of membranes and cytosol and did not exceed 20% of the maximum counts.

As described previously, low molecular weight G proteins with molecular masses of about 20–28 kDa, in contrast to the 40-kDa α subunits of heterotrimeric G proteins, can be detected by their restored ability to bind guanine nucleotides such as [^{32}P]GTP (55) or [^{35}S]GTPγS (47, 48) after blotting and reconstitution on nitrocellulose. We used this technique to visualize low molecular weight G proteins in the membrane and cytosolic fractions, which might account for the observed enhancing effect of GTPγS in our cell-free assembly system and might be affected by B-003. In contrast to recent publications, we used [^{35}S]GTPγS instead of [^{32}P]GTP, for two reasons. Firstly, in agreement with recent observations (55, 56), [^{35}S]GTPγS showed a much better signal-to-noise ratio, in comparison with [^{32}P]GTP, thus leading to an improved resolution of the labeled bands on the blot; secondly, it provided a closer comparison with the guanine nucleotide-binding conditions because in our *in vitro* assembly experiments we used GTPγS as the stimulating nucleotide. Protein blot probing with [^{35}S]GTPγS detected a predominant low molecular weight G protein with a molecular mass of approximately 21 kDa (p21) in the cytosolic fraction (Fig. 8, *right lane*) and a 23-kDa protein in the membrane fraction (Fig. 8, *left lane*). In addition to the cytosolic low molecular weight G protein p21, two additional proteins could be detected in the cytosolic fraction, having molecular masses of 19 kDa and 23 kDa. To clarify the question of whether one of these detected low molecular weight G proteins accounted for the observed strong stimulatory effect of GTPγS in our cell-free system, we performed *in vitro* assembly/co-sedimentation experiments, as described in Experimental Procedures, and, again, used the protein blot technique to visualize the small G proteins. As shown in Fig. 9A, after assembly and sedimentation by ultracentrifugation >70% of the initial GTPγS-enhanced oxidase activity was conserved in the pelleted membranes. Protein blot probing of pelleted GTPγS/AA-assembled oxidase in parallel revealed an approximately 2-fold increase of the cytosolic low molecular weight G protein p21, in comparison with basal conditions, whereas the 23-kDa band remained unchanged (Fig. 9B). The observed increase could be shown to be specific and evidently not due to nonspecific GTPγS/AA-induced precipitation of cytosolic protein, because control incubations using cytosol, AA, and GTPγS without membranes led to only weak sedimentation of the low molecular weight G protein p21 after ultracentrifugation, i.e., about 25–50% of the intensity seen after basal assembly and co-sedimentation (data not shown). Because reisolated membranes without cytosol, after assembly and ultracentrifugation, did not reveal a 21-kDa band, the observed 2-fold increase of the low molecular weight G protein p21 in the pelleted fraction after coincubation of membranes and cytosol very likely is due to a

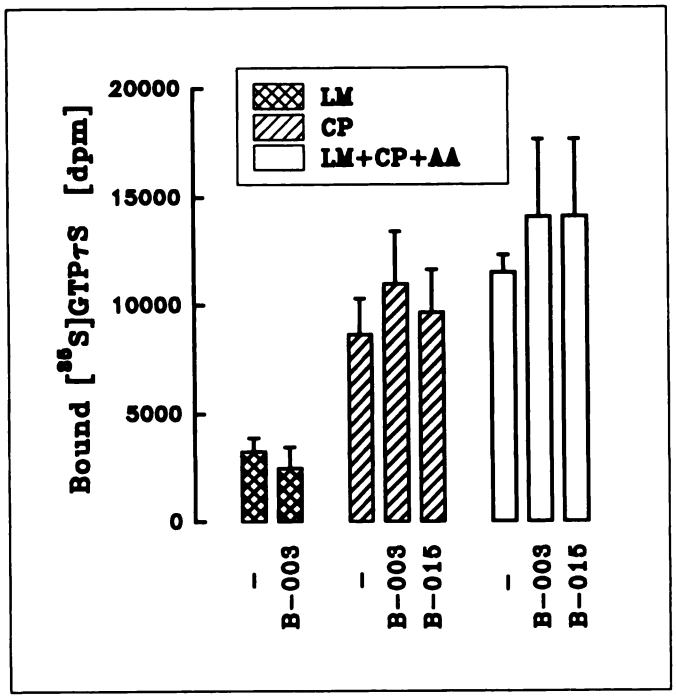


Fig. 7. Influence of B-003 and B-015 on [^{35}S]GTPγS binding to neutrophil membrane and cytosolic fractions. Binding of [^{35}S]GTPγS to membrane (*LM*) or cytosolic (*CP*) protein alone or under assembly conditions (*LM+CP+AA*) was studied in the presence of 20 μM B-003 or 20 μM B-015 (0.4% DMSO), as described in Experimental Procedures. Values are given as means ± standard errors of four to six experiments with cytosolic and membrane fractions from different donors.

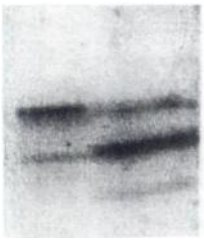


Fig. 8. [^{35}S]GTPγS protein blot probing of membrane and cytosolic G proteins. Proteins of light membranes or cytosol were separated by SDS-PAGE on 12% polyacrylamide gels and were transferred to nitrocellulose sheets. After incubation with [^{35}S]GTPγS and washing as described in Experimental Procedures, bound guanine nucleotide was detected with a PhosphoImager. *Left lane*, 7–10 μg of light membranes; *right lane*, 50 μg of cytosol. A representative blot of four independent experiments is shown.

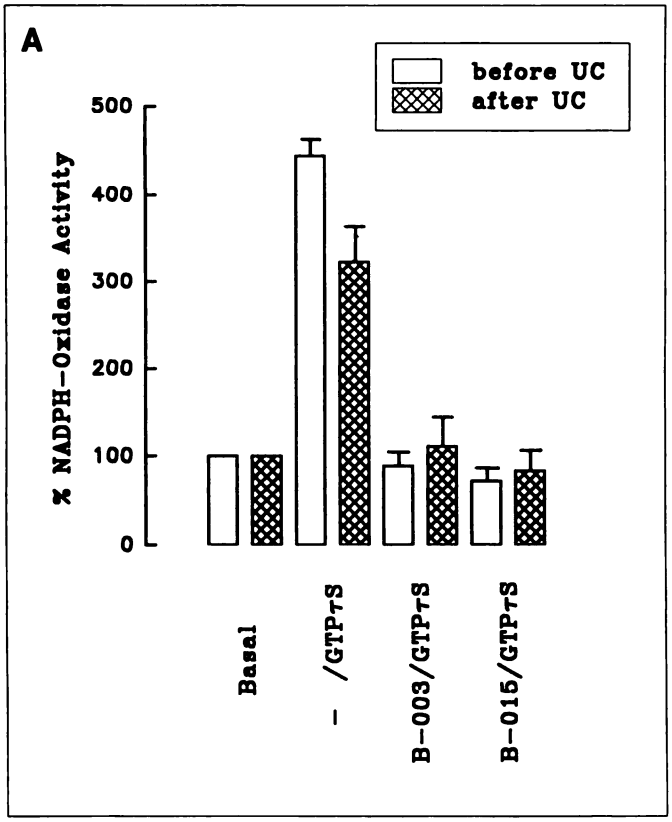


Fig. 9. *In vitro* assembly/co-sedimentation experiment. A, Cell-free NADPH-oxidase activity before and after ultracentrifugation (*UC*). Cell-free oxidase assembly/co-sedimentation was done with light membranes (25 μg) and cytosol (500 μg), as described in Experimental Procedures. Aliquots of the assembly mixture before and after ultracentrifugation were assayed for NADPH-oxidase activity. After monitoring of the baseline, enzyme activity was triggered with 200 μM NADPH and continuously detected as SOD-sensitive cytochrome *c* reduction at 550 nm/538 nm. B, Detection of the GTPγS-stimulated translocation of the cytosolic low molecular weight G protein p21. After *in vitro* assembly/co-sedimentation in the presence or absence of 10 μM GTPγS, aliquots of the sedimented membranes were separated by SDS-PAGE and the protein blot was probed with [^{35}S]GTPγS, as described in Experimental Procedures. *Left lane*, basal; *right lane*, + GTPγS.

GTPγS-AA-induced translocation of the small cytosolic G protein. The specificity of the detected G proteins for guanine nucleotides was demonstrated by the strong displacement of bound [^{35}S]GTPγS by 10 μM unlabeled GTP (data not shown). However, as summarized in Fig. 10, neither B-003 (30 μM) nor B-015 (30 μM) affected this GTPγS-dependent translocation of the low molecular weight G protein p21, under conditions where the activity of the sedimented NADPH-oxidase measured in parallel was inhibited to basal levels.

Finally, we studied the structure-activity relationship of the various ascorbic acid derivatives synthesized in addition to B-003 and B-015 (Fig. 11). For these experiments we used the

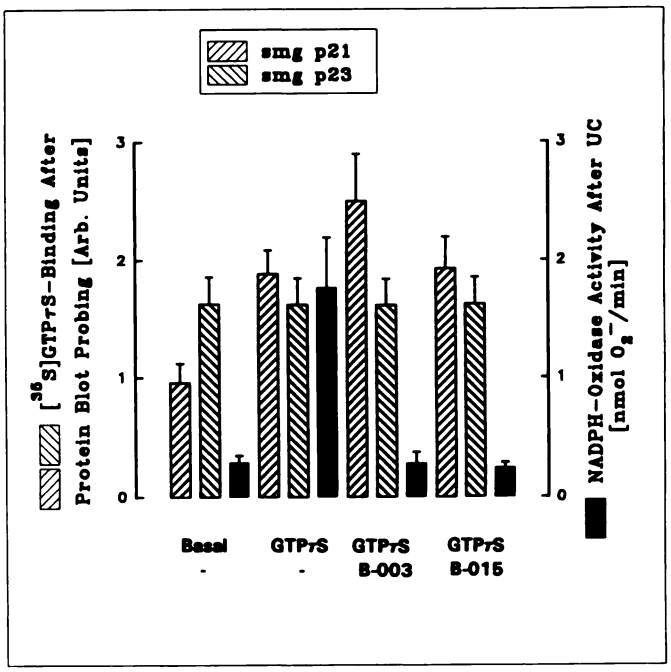


Fig. 10. Effect of B-003 and B-015 on the GTPγS-enhanced translocation of the cytosolic low molecular weight G proteins. After *in vitro* assembly/co-sedimentation, resuspended samples were electrophoresed by SDS-PAGE and transferred to nitrocellulose, and the protein blot was probed with [^{35}S]GTPγS, as described in Experimental Procedures. Where indicated, the assembly mixture was preincubated with 30 μM B-003 or 30 μM B-015 for 2 min before addition of GTPγS and AA.

reconstituted GTPγS-stimulated system but a higher dilution, corresponding to 5×10^6 PMN/ml. For these lower protein concentrations less AA was required, with an optimum at 50 μM (Fig. 11, *inset*). It was determined that lower concentrations of B-003 and B-015 (10 μM) were sufficient to reach maximum inhibition. B-027, the C_{18} chain-length analogue of B-003, was even slightly more efficient, but the shorter chain-length compounds B-005 (C_8) and B-004 (C_{12}) and the 6-*S*-aryl derivative B-028 were less potent or inactive, respectively. Interestingly, the compound B-019 (6-*S*-hexadecyl-2,3-dimethoxyascorbic acid) and the 2,3-dihydroascorbyl-6-palmitoyl ester B-010 were still active but to a much lower degree. From this, we conclude that the strong dependence of inhibition on the chain length was preserved in the reconstituted system but the characteristic large difference between B-003 and B-015 existing with whole cells was lost.

Discussion

Our experiments designed to study the inhibitory mode of action of B-003 in human PMN could not reveal the ultimate target of B-003 and its active analogues, but several clear findings with this interesting group of compounds could be obtained. First, it was fortunate to find that B-015, the regioisomer of B-003, was inactive in whole cells but comparatively active in the cell-free activation system of NADPH-oxidase, although for both compounds much higher concentrations were needed for maximum inhibition of the cell-free assembly. This is in agreement with the previously postulated accumulation of B-003, but not of B-015, in human PMN (4). The basis of this process may be related to the very different pK_a values of the two enolic compounds, which explain the observed low IC_{50}

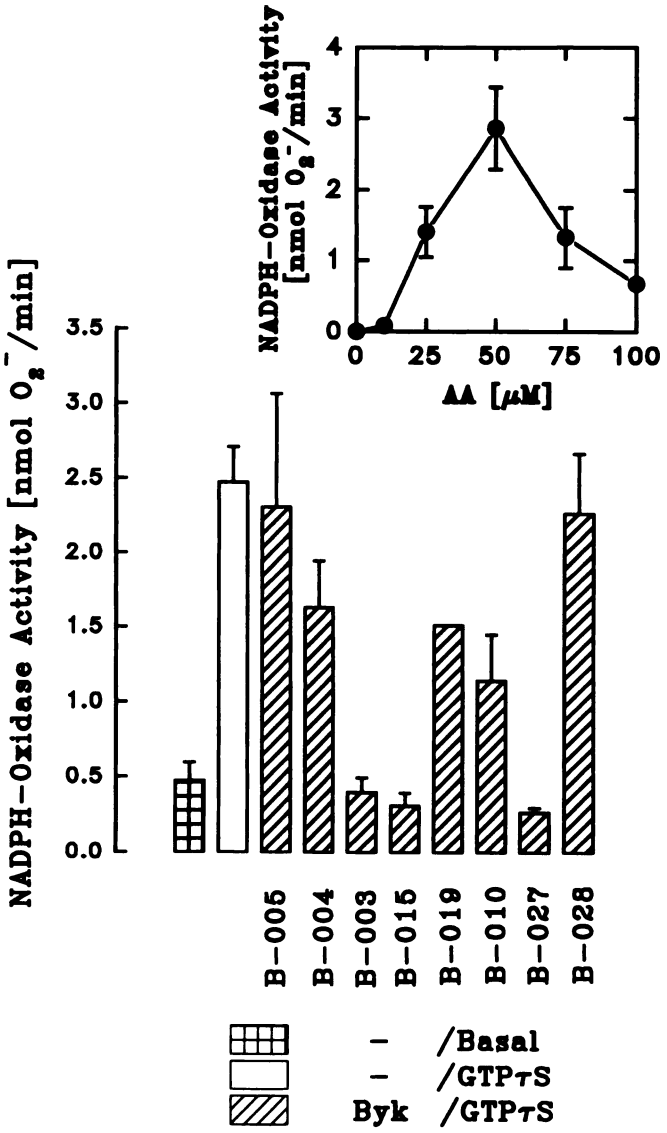


Fig. 11. Effect of structurally different 6-*S*-alkylascorbyl derivatives on the cell-free NADPH-oxidase activity. Neutrophil membranes (3–5 μg) and cytosol (50 μg) equivalent to 5×10^6 cells/ml were preincubated for 2 min at 27° with 10 μM concentrations of the ascorbyl alkyl derivative in the presence of 10 μM GTPγS. NADPH-oxidase assembly was triggered with the optimum concentration (50 μM) of AA (*inset*). After 3 min of assembly, the activity was continuously measured as SOD-sensitive cytochrome *c* reduction, as described in Experimental Procedures. Values are means ± standard errors (three to five experiments). *Byk*, + B –.

values for B-003 in intact PMN. The low pK_a value of the ascorbic acid ring as an important component in the pharmacological efficacy of B-003 has to be separated from a second requirement for inhibitory potency, which depends on a long alkyl side chain with 16 or 18 carbon atoms. Because, however, the ascorbic acid 6-palmitoate ester, the 2-*O*-octadecylether CV-3611, and the 6-*S*-alkylthioethers are active to nearly the same degree, not much selectivity seems to reside in the linkage or the neighboring groups of the side chain.

A second result also facilitated the discussion of the mechanism of action. The surprising lack of any effect of B-003 on the phorbol ester-stimulated oxidative burst in whole PMN or on particulate NADPH-oxidase activity prepared from PMA-pretreated PMN excluded a direct inhibitory effect of the ascorbyl alkyl derivative on the O_2^--producing enzyme system itself. Moreover, the results indicate that an inhibition of the PKC-dependent phosphorylation of the p47-*phox* subunit seems to be unlikely and that the cells were not nonspecifically damaged by the amphiphilic ascorbic acid derivative. Together with the observed lack of effect of B-003 on the receptor-dependent calcium mobilization triggered by two different agonists, the results narrow the mode of action to some target(s) belonging to the Ca^{2+}-dependent signal transduction pathway of NADPH-oxidase assembly, downstream from receptor and G_c activation. A similar locus of inhibition was postulated for the very potent burst inhibitor wortmannin (41, 49), which also blocks the receptor-stimulated pathway but not the PMA-triggered production of superoxide. At variance with the action of wortmannin, however, B-003 potently inhibited the AA-triggered cell-free assembly of the NADPH-oxidase, therefore pointing to a more distal inhibitory mode of action of the ascorbic acid derivative. Interestingly, the inhibitory effect of B-003 on the cell-free NADPH-oxidase system is similar to that of the synthetic peptide RGVHFIF, corresponding to the cytoplasmic domain of the large subunit of cytochrome b_{558}, which inhibits only the AA-induced assembly of the complex and not the fully assembled and catalytically active enzyme complex (57, 58). Tentatively, our results suggest that B-003, similarly to RGVHFIF, inhibits processes critical to oxidase activation, rather than electron-transfer processes. Because, however, RGVHFIF, at variance with the effect of B-003, has been shown to inhibit both fMLP- and PMA-triggered superoxide production (59) and, on the other hand, the translocation of the essential oxidase component p47-*phox* to the membrane has been described after PMA activation (28), an interaction of the selective burst-inhibiting 6-*S*-hexadecylascorbate derivative with the essential function/translocation of the 47-kDa oxidase subunit seems to be unlikely. Because of this, other essential components of the NADPH-oxidase, such as the recently described *ras*-like G proteins, seemed to be more likely targets of B-003. This idea was supported by the prounced inhibitory effect of the 6-*S*-hexadecylascorbate derivatives when the cell-free oxidase system was incubated with stimulatory guanine nucleotides. Surprisingly, neither the binding of the activating guanine nucleotide to possibly involved low molecular weight G proteins nor the binding of the detected G proteins to the membranous fraction seemed to be affected by B-003, under conditions where the GTPγS-stimulated oxidase activity was inhibited to basal levels. It is of interest that similar results have been obtained with the heptapeptide RGVHFIF, which, although strongly inhibiting the cell-free oxidase activity, did not affect the translocation of the cytosolic components (57).

Thus, at present the ultimate target and the inhibitory mode of action of the acorbic acid derivatives remain speculative. If, however, AA or another fatty acid-like molecule is involved in the assembly process, one could imagine the amphiphilic ascorbate derivatives interfering, with their C_{16}-C_{18} alkyl side chains, with the postulated fusiogenic function of these physiologically relevant fatty acids. On the other hand, looking at the structure of the long chain alkyl ascorbyl derivatives, there could also be a connection to the recently described lipophilic cell-free inhibitor of NADPH-oxidase activity, with a proposed structure of propionic acid 3,3′-thiobis(didodecyl)ester. This deactivator, which has been isolated from PMN homogenates, is speculated

to inhibit the cell-free oxidase assembly by interfering with the essential function of the carboxyl-terminal isoprenoid moiety of involved ras-like proteins (59). Accordingly, possible targets for the examined long alkyl chain ascorbic acid derivatives could be not only membranes but also binding proteins for fatty acid-modified or isoprenylated components of the system, which have been shown recently to modulate the activity of the cell-free NADPH-oxidase system (37).

Finally, it is intriguing to assume a mechanistic link between such processes and the events leading to the simultaneous inhibition of secretion in PMN and the observed release of GSH.[1] The latter did not occur after microtubule disassembly by nocodazole, and it may be determined that all processes observed with B-003, CV-3611, or ascorbyl-6-palmitoate are related to altered dynamics of the cytoskeleton.

[1] D. Roth, unpublished experiments.

Acknowledgments

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