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THE SUPPRESSION OF GRANULOCYTE FUNCTIONS BY LIPOPHILIC ANTIOXIDANTS

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Abstract—The effects of several antioxidants on the three major functions of human neutrophils—oxidative burst, secretion and leukotriene formation—were investigated with special emphasis on the lipophilicity. The most striking differences were obtained when ascorbate and the lipophilic ester ascorbyl palmitate were compared. As expected, the luminol- and lucigenin-dependent chemiluminescence was inhibited by all antioxidants to a different degree. Ascorbyl palmitate was able to block the biphasic luminol-dependent response completely with IC_{50} values of 10 and 25 μM for the first and second phase, respectively. In contrast, ascorbate only blocked efficiently the first phase of the response.

The secretion of elastase was inhibited by ascorbyl palmitate dose-dependently with an IC_{50} value of around 200 μM, whereas ascorbate was completely inactive. Electron microscopy supported the assumption that inhibition was due to a block in degranulation and not to enzyme inactivation. This was further supported by a parallel, although somewhat lower, inhibition of other secretory enzymes like myeloperoxidase, β-glucuronidase or lysozyme.

Cells treated with the Ca^{2+}-ionophore A23187 responded by LTB_4-synthesis which was also inhibited by ascorbyl palmitate. A very efficient inhibition was observed in cell homogenates with an IC_{50} value of 1.5 μM. No inhibition by ascorbate was detected in both systems. Concomitant with the inhibition of 5-lipoxygenase the activity of 15-lipoxygenase increased.

We conclude that cellular reductants may control neutrophil functions and that the inhibition by ascorbyl palmitate of the three processes relevant for inflammatory responses could be of therapeutic importance.

Inflammation is characterized by a massive accumulation of polymorphonuclear leukocytes (PMN)* which have the function of phagocytizing and degrading organic material in body tissues [1]. This process requires a sophisticated machinery of signal perception, chemotaxis, phagocytosis, secretion of oxygen radicals and hydrolytic enzymes and, finally, the release of leukotriene B_4 (LTB_4) as a chemotactic second wave messenger [2]. Although being part of the body's defense system and as such beneficial and even essential, there are common pathological conditions like shock, chemical irritation or autoimmune responses including chronic inflammatory diseases which cause undesired activation of the inflammation response [3]. The three main steps in this chain of events have been suggested as promising targets of pharmacological intervention: (i) the formation of oxygen radicals as a possible source of endothelial damage, (ii) the secretion of hydrolytic enzymes, notably elastase, being able to attack normal tissues, and (iii) the biosynthesis of LTB_4 as the most potent chemoattractant for PMN [4, 5].

With regard to the formation of oxygen radicals, beneficial effects of superoxide dismutase and catalase have been reported in the treatment of a variety of inflammatory diseases [6, 7]. Antioxidants like vitamin E were also found to be useful but high doses may be required [8]. Considering that the oxidizing oxygen radicals are generated in cellular phagosomes as well as in the extracellular space, it seemed worthwhile to study once more the protective effects of antioxidants as a function of their lipid solubility. This was done by selecting fatty acid esters of known watersoluble antioxidants and studying their effect on PMN elicited chemiluminescence [9, 10].

The secretion of various types of granules is another important response of the PMN to stimulation. Systematic research on the influence of antioxidants on the secretory process has not been reported. In order to determine whether antioxidants can interfere with this event, we have measured the release of secretory enzymes and in addition have looked morphologically at the disappearance of the granules.

The inhibition of leukotriene B_4 formation by PMN has been regarded as an attractive pharmacological target. Interestingly, many of the drugs known to interfere with 5-lipoxygenase such as quer-

* Abbreviations used: Asc, L-ascorbic acid; Asc-P, L-ascorbyl palmitate; BSA, bovine serum albumin; CE-S, chromanol ethanol stearate; CL, chemiluminescence; DHC-S, dihydrocaffeic acid stearate; DMSO, dimethylsulfoxide; EDTA, ethylenediamine tetraacetic acid; FMLP, *N*-formyl-methionyl-leucyl-phenylalanin; HETE, hydroxyeicosatetraenoic acid; LT, leukotriene; NADPH, nicotinamide adenine dinucleotide phosphate; PG, prostaglandin; PBS, phosphate buffered saline; PMN, polymorphonuclear leukocytes; Toc, α-tocopherol; Toc-Ac, α-tocopherol acetate; Toc-Nic, α-tocopherol nicotinate.

Enzymes: Elastase (EC 3.4.21.11), β-D-glucuronidase (EC 3.2.1.31), lactate dehydrogenase (EC 1.1.1.27), 5-lipoxygenase (EC 1.13.11.34); 15-lipoxygenase (EC 1.13.11.12); lysozyme (EC 3.2.1.17); myeloperoxidase (EC 1.13.11.12); NADPH-oxidase (EC 1.6.99.6).

cetin, nordihydroguaiaretic acid (NDGA) or BW 755C possess antioxidant properties [11, 12]. As a physiological antioxidant, tocopherol was found to inhibit 5-lipoxygenase activity and also to suppress random migration and chemotaxis of PMN [13, 14]. Ozaki *et al.* [15] compared several compounds with regard to their ability to affect LTB_4 synthesis, degranulation and oxygen radical formation. Since no close correlation existed between these events, an LTB_4 modulated PMN function was excluded.

In view of the inhibitory actions of tocopherol on various functions of PMN, we have studied in this paper the influence of lipophilicity of several antioxidants on oxygen radical formation, secretion and 5-lipoxygenase activity. Tocopherol was chemically modified at its functional OH-group by esterification with acetic acid and nicotinic acid in order to have controls with non-reducing properties. Dihydrocaffeic acid and chromanol ethanol were employed as stearate esters. The most impressive example for hydrophilic versus hydrophobic antioxidants was represented by the couple ascorbate-ascorbyl palmitate.

MATERIALS AND METHODS

Reagents

Luminol, lucigenin, trypsin inhibitor and ATP (adenosine 5'-trisphosphate) were purchased from Boehringer Mannheim (Mannheim, F.R.G.); ethyl acetate (Spectranal^R) from Riedel-de Haen (Hannover, F.R.G.); methoxysuccinyl-L-Ala-L-Pro-L-Val-7-amino-4-methylcoumarin from Bachem Feinchemikalien (Bubendorf, Switzerland); arachidonic acid from Larodan (Malmö, Sweden); LTB_4 was a generous gift of Dr Bartmann (Höchst AG); w-Hydroxy-LTB_4, w-carboxy-LTB_4, 5-, 12- and 15-HETEs and 5,6-diHETEs were obtained from Paesel (Frankfurt, F.R.G.); LTC_4, LTD_4 and LTE_4 from Ultrafine Chemicals (Salford, U.K.) and Ficoll-Paque from Pharmacia GmbH (Freiburg, F.R.G.). The LTB_4-isomers were kindly provided by Dr Haurand (Grünenthal GmbH, Aachen, F.R.G.); Cremophor EL and the antioxidants were gifts from Knoll AG (Ludwigshafen, F.R.G.). Solvents for HPLC analysis were HPLC or analytical grade and were purchased from Merck (Darmstadt, F.R.G.). All other chemicals were purchased from the Sigma Chemical Co. (Deissendorf, F.R.G.).

Cell isolation and degranulation assays were performed in phosphate buffered saline (PBS). A Hepes-buffered Krebs medium containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, 1 mg/ml glucose and 1 mM $CaCl_2$ was used for CL measurements; pH was adjusted to 7.4, ionic strength being 280 mOsm. Zymosan was opsonized in 50% human serum as described in the literature [16].

The antioxidants (40 μM stock solutions) were kept in aqueous solution with Cremophor EL (0.21 g/ml). This product is commercially available (BASF, Ludwigshafen, F.R.G.) as the condensation product of 1 mol of castor-oil with 35 mol ethylene oxide. Ten millilitres of pre-warmed H_2O were added slowly under mechanical stirring to a solution of the antioxidant in 2.5 g Cremophor EL kept at 60–65° under nitrogen gas. The resulting clear solutions were filtered and stored in portions under nitrogen at 4°. Precipitates formed during storage were redissolved at 37°.

PMN isolation

Freshly drawn venous blood (200 ml) from healthy adult donors was supplemented with 0.38% sodium citrate. Neutrophils (PMN) were obtained by a modification of the Ficoll-Paque method [17]. In brief, platelet-rich-plasma (PRP) was separated by a 20 min centrifugation step at 200 *g*. Red blood cells were sedimented in 1% Dextran for 30 min; 25 ml of the resulting supernatant were layered on top of 10 ml Ficoll-Paque (containing 0.25% BSA) and the tubes were centrifuged at 525 *g* for 15 min. The resulting pellet was washed with 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) and centrifuged for 5 min at 400 *g*. Addition of hypotonic buffer (0.83% NH_4Cl, 0.1% $KHCO_3$, 0.0037% EDTA) caused lysis of remaining red blood cells during standing for 6 min at 4°. PMN were washed twice in PBS and resuspended to obtain 2×10^7 cells/ml and stored at 4°. The purity of the PMN was about 95% and the viability as measured by Trypan Blue exclusion and LDH-release was greater than 97%.

For chemiluminescence measurements, cells 10^6 ml^{-1} were resuspended in Hepes-buffered Krebs medium without glucose and $CaCl_2$; for degranulation assays the cell number was adjusted to 2×10^7 ml^{-1} in PBS.

Chemiluminescence assay

The measurements were performed with a prototype Luminescence Emission Analyzer (LEA) constructed by Raytest, Straubenhardt, F.R.G. The instrument was equipped with a Hamamatsu photomultiplier tube type R268 cooled to −5°, working in photon-counting mode. Assays were performed in disposable Petri dishes (Nunc) with 35 mm diameter normally at 37°. Some experiments were performed at 25°.

Cells (10^5) with luminol (5 μM) or 5×10^5 cells with lucigenin (20 μM) as enhancers were incubated in Hepes-buffered Krebs medium containing 0.05% bovine serum albumin (BSA) in Petri dishes at 37°. After 4 min the stimulus FMLP (0.5 μM) or opsonized zymosan (0.5 mg/ml) was added. The emission obtained during preincubation was generally much lower than 5% of the stimulated response, normally being around 1%. In contrast, when the cells were incubated without BSA in the presence of luminol or lucigenin, an appreciable emission was obtained after a lag phase of about 1 min. The response to stimulation with FMLP or zymosan was strongly diminished and sometimes absent. The "unstimulated activation" was not caused by the enhancer but probably by contact of the cells to the plastic surface of the Petri dishes. It was inhibited by BSA in a concentration-dependent manner, 0.05% normally being sufficient for complete inhibition.

The antioxidants were added to the medium immediately before the incubation period. Control runs with Cremophor EL (2.1 mg/ml) showed slightly higher maximal emissions than without; how-

ever, the kinetics remained the same. Maximum intensities, as well as the integrals of the emission, were taken for evaluation. Both methods yielded practically identical results. The antioxidant influence was expressed as percent inhibition of the maximum emission intensity relative to control values obtained in the presence of Cremophor EL.

Myeloperoxidase–H_2O_2-chemiluminescence

A cell-free system was used to investigate the antioxidant influence on the myeloperoxidase–H_2O_2 reaction [18]. Supernatants of sonified and Triton X-100-treated (0.1% Triton X-100, 10 min at 37°) cells (corresponding to 10^5 ml^{-1}) were stimulated with 2.5 μM H_2O_2 in the presence of 5 μM luminol. Antioxidants were added in Cremophor EL before the addition of H_2O_2. The initial intensity of the chemiluminescence emission was used for quantitation.

Degranulation

PMN (2×10^6 ml^{-1}) preincubated in PBS containing 0.05% BSA, 1 mM Ca^{2+}, and 2.5 μg/ml cytochalasin B at 37° for 5 min were stimulated with FMLP (0.5 μM). After an additional 5 min, the samples were centrifuged (10,000 *g*) and supernatants were separated. Antioxidants were added before preincubation. 2×10^5 PMN ml^{-1} were used in both assays in experiments where chemiluminescence and degranulation were compared directly.

Enzyme assays

Lactate dehydrogenase (LDH) as a marker of cell viability, was determined in the cell supernatants as described in the literature [19]. The release was generally lower than 5% of the total content. Incubation with Cremophor EL led to a decreased LDH release pointing to a stabilisation of the cells by the cremophor. Antioxidants did not show any effect on the release of this enzyme.

Elastase activity was determined by a variation of a method given in the literature [20]. Two hundred microlitres of the supernatant of stimulated cells were incubated with 2×10^{-5} M methoxysuccinyl-L-Ala-L-Ala-L-Pro-L-Val-7-amino-4-methyl-coumarin in PBS in 1 ml total volume at 37° for 5 min. The reaction was stopped by addition of 10 μl trypsin inhibitor (10 mg/ml) and the product 7-methyl-coumarin determined fluorometrically (excitation: 313 + 336 nm/Hg; emission: 430–3000 nm).

β-Glucuronidase and lysozyme were assayed according to literature methods [21, 22]. Cremophor EL and the antioxidants did not interfere with any of the enzyme assays described above. Maximal stimulation by FMLP caused the release of about 70% of the lysozyme content and of about 50% of the total glucuronidase. The 100% values were obtained by sonification of Triton X-100-treated cells. This method could not be used for elastase probably due to interference with the simultaneously liberated α_1 protease inhibitor.

Myeloperoxidase was determined by a chemiluminescence assay with luminol as enhancer [18]. Ten microlitres of the cell supernatant incubated in the presence of luminol (5 μM) in a total volume of 2.0 ml Hepes-buffered Krebs medium at 37° were stimulated by the addition of H_2O_2 (5 μM final concentration). Initial chemiluminescence intensities were used for evaluation. As antioxidants interfered with the assay, appropriate control experiments were performed for each concentration of antioxidant used in the degranulation assay.

Electron microscopy

Cells were fixed in 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer pH 7.0, 15 min, 0°. They were then washed in the same buffer (3 × 10 min, 0°), postfixed for 1 hr in 1% (w/v) osmiumtetroxide in the same buffer, and then washed again as above (0°). Dehydration was performed at room temperature using graded acetone series of 50, 70, 90, 95 and 3 × 100% aceton, each for 10 min. Samples were embedded in Spurr's low viscosity resin and polymerized for 2 days at 70°. Ultrathin sections of 40 nm thickness were prepared with a Reichert-Jung ultramicrotome Ultracut E. Sections were stained for 1 min with 1% aqueous uranylacetate and then for 1 min with alkaline lead citrate pH 12.0. Micrographs were taken at 80 kV acceleration in an electron microscope type Zeiß EM 10.

Arachidonic acid metabolites

Incubations with whole cells. Cell suspensions (10^7 PMN ml^{-1}) were preincubated for 5 min at 37° with 1 mM Ca^{2+} and the given concentrations of Asc-P, palmitate and Asc. Asc-P and palmitate were dissolved in DMSO and suspended in buffer before addition to the cell suspension. All assays contained 0.5% DMSO.

The cell suspensions were then stimulated with 1 μM A23187. After a 5 min incubation at 37°, 0.9 ml were transferred into 4 ml of ethyl acetate supplemented with 0.1 ml of 0.09 M HCl and 100 ng of PGB_2 as an internal standard and the lipoxygenase metabolites were extracted at a resulting pH of 3.

Incubations with 10,000 g *supernatants.* Ten ml of a cell suspension containing 10^8 cells in PBS were sonified at 4° (4 × 10 pulses at microtip energy limit) and centrifuged for 5 min at 10,000 *g*. One-millilitre aliquots of the supernatants were preincubated for 5 min at 37° with 1 mM Ca^{2+}, 1 mM ATP and the indicated concentration of Asc-P, palmitate and Asc, respectively. All assays contained 0.5% DMSO.

The reaction was then started by the addition of 20 μM arachidonate (diluted 1:100 from a 2 mM stock solution in 0.01 M NaOH), incubated for 5 min at 37° and 0.9 ml stopped as given above.

Reverse phase HPLC-analysis. HPLC of the arachidonate metabolites was performed using a 2150 HPLC pump, a 2151 HPLC controller, a 2156 solvent conditioner and a 2151 variable wave length monitor from LKB (Gräfelfing, F.R.G.). Wave length setting was at 280 nm for the leukotrienes and at 237 nm for HETEs. A Nucleosil ODS silica (250 × 4.6 mm, 5 μm particle size) prepacked column and an adequate guard column (10 × 4.6 mm) from Bischoff (Leonberg, F.R.G.) were used for separation. The samples were applied to the column using a 100 μl injection loop and a Rheodyne (Cotati, CA) injection valve, type 7125. The column was eluted at room temperature with solvent A (acetonitrile 25, methanol 40, water 34, acetic acid 0.3, phosphoric acid 0.2, ammonia 0.3 (v/v)) and solvent B (aceto-

nitrile 40, methanol 29, water 30, acetic acid 0.5, phosphoric acid 0.2 and ammonia 0.3 (v/v)). The apparent pH of solvent A was measured as 3.9, for solvent B 3.65. The solvents were filtered through 0.45 μm membrane filters (Schleicher & Schuell, F.R.G.) before use and continuously gassed with helium. At a flow rate of 1.0 ml/min, the gradient was started with 100% solvent A for 3 min, increased to 100% solvent B within 12 min and continued with solvent B up to 34 min. The column was then washed with methanol. Peak curves were calculated by means of a Merck-Hitachi integrator (Darmstadt, F.R.G.) and the amount of metabolite was determined from a standard curve with PGB_2. Extinction coefficients for PGB_2 at 280 nm of 28,650 M^{-1}, for the trienes at 280 nm of 40,000 M^{-1} and for HETEs at 237 nm of 30,500 M^{-1} were used. Identification of the different metabolites was made by comparison with the retention times of purchased standard substances.

RESULTS

1. Luminol- and lucigenin-enhanced chemiluminescence

A typical biphasic increase in CL was observed upon stimulation of human neutrophils with the chemotactic peptide FMLP in the presence of luminol. At 37°, the weaker first peak only appeared as a shoulder on the more intense second peak (Fig. 1a). If the reaction temperature was lowered to 25°, both phases became clearly separated and the effect of inhibitors could be studied for each phase [23]. A monophasic response was observed with lucigenin as a chemiluminescence enhancer (Fig. 1b). In both systems, antioxidants suppressed this burst of CL (Figs 1a and b), which could be quantitated as the integral of the emitted light or simply as the maximum height of the CL peak. In general, we used the latter method in favour of a shorter reaction time (see under Materials and Methods).

Figure 2 shows the inhibition of FMLP-stimulated CL (second peak, I_2) at 40 μM concentrations of several antioxidants. The luminol-enhanced CL was in all cases inhibited more efficiently than the lucigenin-derived CL, the differences being significant for Asc, chromanol ethanol stearate and tocopherol. Asc-P was most effective in both assays, although with luminol Asc showed a similar efficiency. The results were obtained with three different cell preparations. The standard deviations, especially with Asc, were high but due to individual differences since the variations within one population were small. In agreement with the non-reducing properties of tocopherol acetate and tocopherol nicotinate, these two compounds were hardly inhibitory in this system. In both tocopherol derivatives, the oxidizable OH-group is blocked by ester linkage, which does not seem to be hydrolyzed significantly under our experimental conditions.

If zymosan was used as a stimulus, the inhibitory activity of all compounds was drastically diminished. Owing to poor reproducibility of the results with different cell preparations, only a representative example is given (Fig. 3).

Concentrating on Asc and Asc-P, we first established a concentration dependence of its inhibitory action on FMLP-stimulated PMN-CL enhanced by luminol. Figure 4 gives one representative example of six independent experiments. In the low concentration range (up to 10 μM) both derivatives appear equally efficient. However, Asc-P above 200 μM inhibits the CL completely, whereas Asc can only block around 50% of the light emission, irrespective of the high concentrations employed. The IC_{50} value for Asc-P was determined as 25 ± 12 μM (mean ± SD, N = 7). At 150 μM Asc-P, generally more than 95% inhibition was achieved, the concentration for complete inhibition was around 250 μM. For Asc, no IC_{50} values could be given because maximum inhibitions varied between 20 and 80% and on average not more than 50% (±25%, mean ± SD; N = 6) of the total CL could be blocked.

To evaluate the effect of Asc and Asc-P on the first peak of the luminol-dependent FMLP- stimulated PMN-CL, experiments were performed at a lower temperature (T = 25°) where the two phases separated clearly. Figure 5 shows a representative

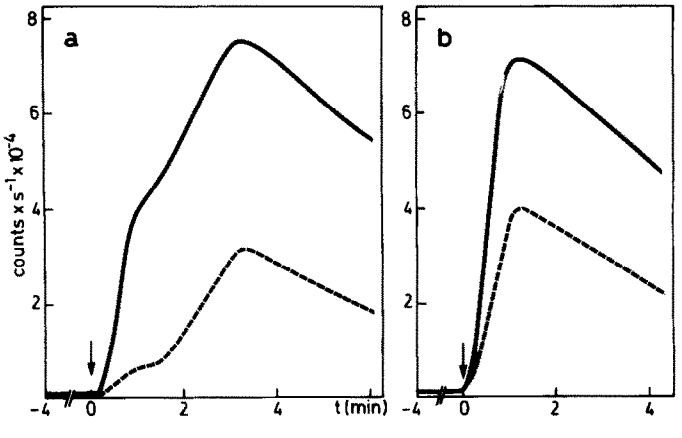

Fig. 1. Chemiluminescence emmission from FMLP-stimulated PMN. (a) Luminol-enhanced emission: the dashed curve was observed in the presence of 40 μM Asc-P. (b) Lucigenin-enhanced emission. The dashed curve was obtained in the presence of 40 μM Asc-P (for conditions see Materials and Methods).

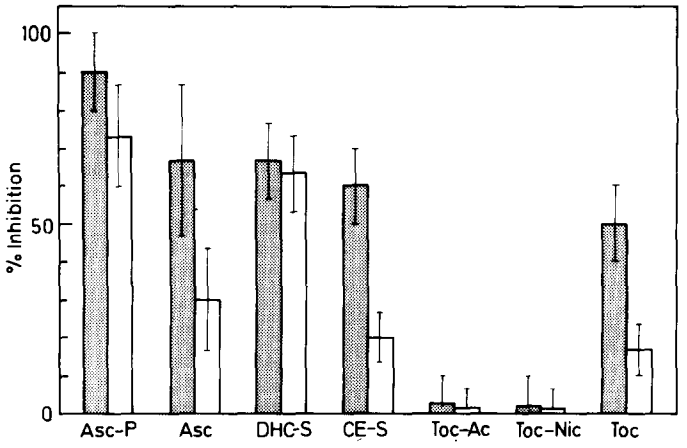

Fig. 2. Antioxidant (40 μM) effect on the FMLP induced luminol- and lucigenin-dependent chemiluminescence. Full columns: luminol-enhanced CL; open columns: lucigenin-enhanced CL. Mean values and standard deviation (σ_{n-1}) from the results of three independent series of experiments are given for luminol and lucigenin, respectively (for conditions see Materials and Methods).

example. The first peak was inhibited by Asc-P with an IC_{50} of about 10 μM as well as by Asc ($IC_{50} \approx$ 20 μM). 100 μM of Asc-P inhibited the first phase completely, whereas Asc showed a maximum inhibition of only 75%. Asc-P blocks the second peak with an $IC_{50} \approx 40$ μM which is in agreement with the results obtained at 37°. The maximum inhibition of the second peak with Asc was only 20%.

In a separate set of experiments it was established that, in a cell-free and soluble system with myeloperoxidase and H_2O_2, Asc and Asc-P had about the same inhibitory effect on luminol-enhanced chemiluminescence ($IC_{50} \approx 2$ μM). Also, all other antioxidants investigated showed significant inhibitory activity in this cell-free system, and the tocopherol derivatives proved to be inactive as expected (data not shown). A comparison of the CL assay with the superoxide-dependent cytochrome *c* reduction assay was not possible due to the direct interaction of several antioxidants with cytochrome *c*.

2. Secretion

The release of the content of secretory granules into the extracellular space constitutes the second important event in the activation of PMN. Elastase, β-glucuronidase, lysozyme and myeloperoxidase were selected as representative enzyme markers for the different types of granules. In the range up to 400 μM and even higher, Asc did not show any inhibitory effect on the FMLP-stimulated secretion of these enzymes; however, Asc-P concentration-dependently blocked the enzyme activities in the supernatants to different degrees. Figure 6 shows a representative example of several independent experiments. 400 μM Asc-P blocked the secretion of elastase by 90 ± 5% (N = 20), of glucuronidase

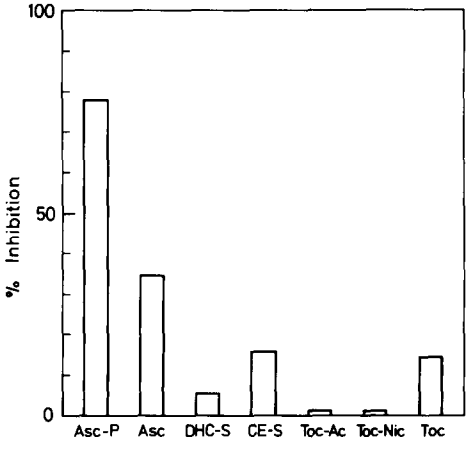

Fig. 3. Antioxidant (400 μM) effect on the zymosan-induced luminol-dependent chemiluminescence. Duplicate experiments performed with one cell preparation (for conditions see Materials and Methods).

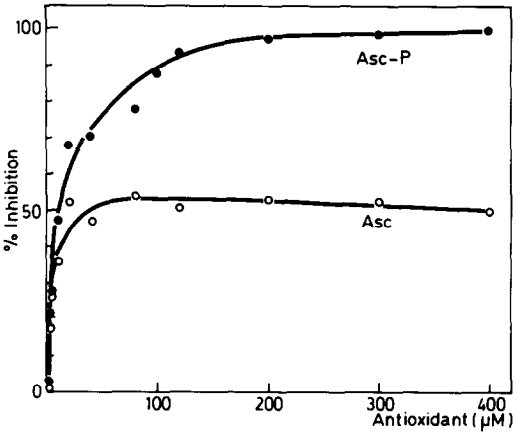

Fig. 4. Effect of ascorbyl palmitate and ascorbate on FMLP-induced luminol-dependent chemiluminescence. Duplicate experiments performed with one cell preparation (for conditions see Materials and Methods).

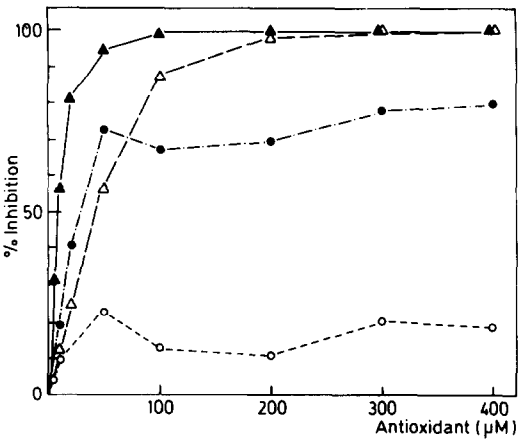

Fig. 5. Effect of ascorbyl palmitate and ascorbate on FMLP-induced luminol-dependent chemiluminescence at 25°. Inhibition of the first (●) and second peak (○) by Asc. Inhibition of the first (▲) and second peak (△) by Asc-P (for conditions see Materials and Methods).

70 ± 20% (N = 6) and of lysozyme 60 ± 10% (N = 6). From the other antioxidants under investigation, none showed more than 20% inhibition at 400 μM (data not shown).

We could confirm by electron microscopy and negative staining of PMN treated with and without Asc-P that, indeed, the degranulation was blocked and not a secondary event like a possible oxidative activation of the secretory enzymes (Fig. 7). In resting neutrophils, the granules are clearly visible as darker spots (Fig. 7a), whereas after stimulation with FMLP in the presence of cytochalasin B the cells are depleted from granules (Fig. 7b). If the stimulation was performed in the presence of 400 μM Asc-P, no significant depletion from granules could be observed (Fig. 7c).

A comparison of the effects of Asc-P on CL emission and degranulation points to a clear quantitative difference in inhibition of both events. The IC_{50} of around 200 μM for the inhibition of elastase degranulation is one order of magnitude higher than that for the inhibition of luminol-enhanced CL. Since we routinely performed the two assays with different numbers of cells, some additional experiments were designed to measure degranulation and luminol enhanced CL under identical conditions in parallel. Figure 8 shows a representative example of the effects of Asc-P and Asc on the two cellular events. Inhibition of degranulation (elastase assay) was not yet observed with 40 μM Asc-P but the luminol-enhanced CL was already inhibited by 70%. An ascorbate concentration between 200 and 400 μM inhibited the CL by 60%, whereas no inhibition of degranulation was noticed.

The application of Cremophor EL in all assays did not interfere with the assays since suspensions in buffer or the use of DMSO or ethanol stock solutions of the antioxidants yielded similar results in the CL and degranulation assays.

3. *Leukotriene formation*

The synthesis of the chemotactic leukotriene B_4 by PMN must be considered as a potent secondary stimulus for inflammation since it leads to a massive accumulation of circulating PMN at the site of a strong stimulus. In order to determine the total 5-lipoxygenase activity in PMN, it is necessary to determine not only LTB_4 but also its isomers, its oxidation products w-hydroxy and w-carboxy-LTB_4, and 5-HETE. This was achieved by HPLC-separation and quantitation.

Since the physiological stimuli leading to LTB_4-formation in intact PMN cannot yet be exactly defined, the Ca^{2+}-ionophore A23187 was used to activate 5-lipoxygenase [24, 25]. Under these conditions Asc-P was able to inhibit the formation of 5-lipoxygenase products in a concentration and time-dependent fashion. Asc, as well as palmitate and a combination of both, proved to be inactive in control experiments (Fig. 9).

According to the observed increase of inhibition with incubation time, a permeation process could be involved. Therefore, homogenates from PMN were prepared, to which 20 μM arachidonate were added. When 5-lipoxygenase was activated by the addition of Ca^{2+} and ATP, Asc-P very effectively inhibited its activity with an IC_{50} value of 1.5 μM (Fig. 10). Neither Asc nor palmitate or both together showed significant inhibition up to 100 μM. It was interesting to note that the 15-lipoxygenase activity, also present in PMN, was stimulated by Asc-P obviously at the expense of 5-lipoxygenase products (Fig. 11).

DISCUSSION

Our results demonstrate that all three characteristic functions of PMN—oxidative burst, secretion and leukotriene formation—are sensitive towards antioxidants, provided that they are lipophilic. The best and most clear-cut results were obtained with Asc-P in comparison with Asc. At 400 μM the lipophilic derivative of vitamin C inhibited completely

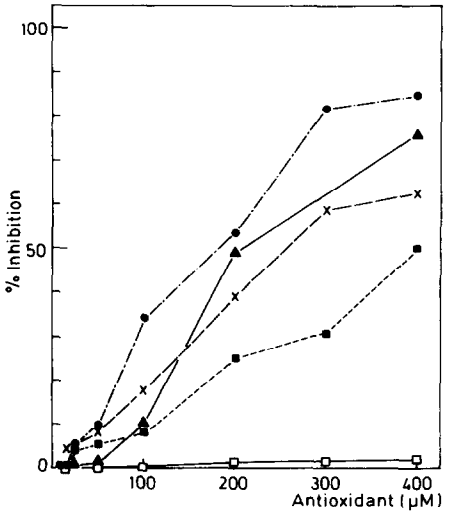

Fig. 6. Effect of ascorbyl palmitate and ascorbate on PMN enzyme secretion. Inhibition of enzyme secretion by Asc-P: ●, elastase; ▲, myeloperoxidase; ×, lysozyme; ■, glucuronidase. Inhibition of enzyme secretion by Asc: □, elastase, myeloperoxidase, lysozyme or glucuronidase (for conditions see Materials and Methods).

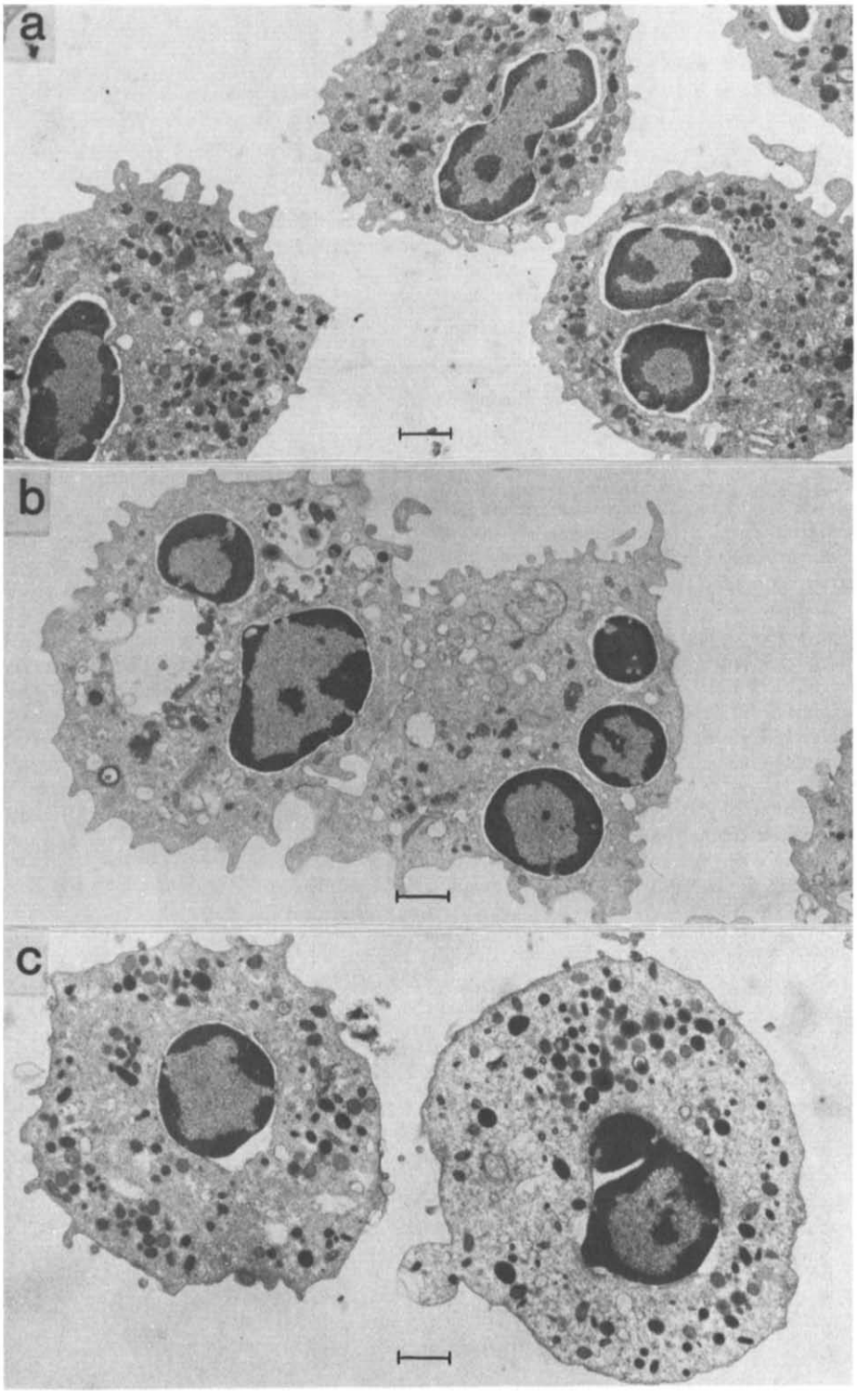

Fig. 7. Electron microscopic evaluation of the effect of ascorbyl palmitate on PMN degranulation: (a) resting cells; (b) FMLP-stimulated cells; (c) cells stimulated in the presence of 400 μM Asc-P. The scale bars indicate 1 μm (for conditions see Materials and Methods).

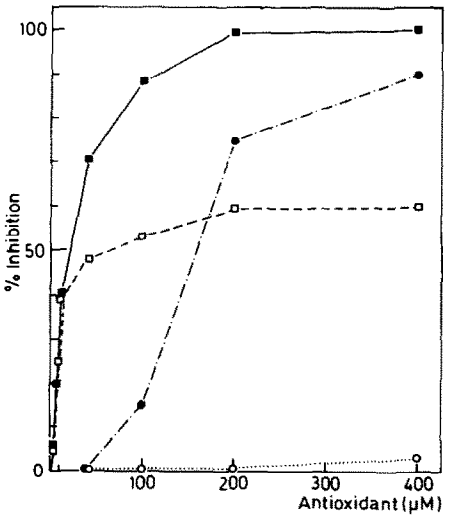

Fig. 8. Simultaneous measurement of FMLP-induced luminol-dependent CL and degranulation in the presence of ascorbate and ascorbyl palmitate. Inhibition of CL (□) and degranulation (○) by Asc. Inhibition of CL (■) and degranulation (●) by Asc-P. Degranulation was measured by the elastase assay (for conditions see Materials and Methods).

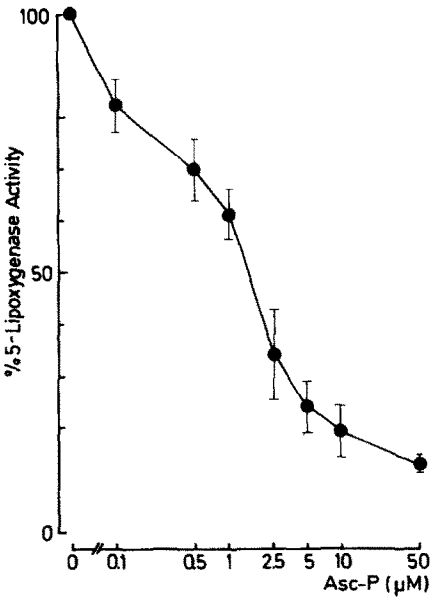

Fig. 10. Effect of ascorbyl palmitate on 5-lipoxygenase activity in PMN homogenates. Values are given as means ± SEM from 5 independent experiments. 100% corresponds to 1390 ± 210 ng (for conditions see Materials and Methods).

not only the oxygen radical induced CL but also to a large extent the secretion of granules and the leukotriene B_4 formation. Although one could presume a common underlying redox regulation of the three major PMN functions, our present knowledge on the mechanism of these three events does not favour such an assumption.

The oxidative burst of PMN reflects the rapid onset of superoxide anion formation after stimulation by FMLP or opsonized zymosan by NADPH-oxidase [26]. Although superoxide radicals possess only weak oxidizing properties, their dismutation to H_2O_2 in conjunction with the secreted myeloperoxidase generates hypochlorite which serves to oxidize phagocytized material. Asc and tocopherol are known to scavenge superoxide radicals [27, 28] and Asc was reported to inhibit the myeloperoxidase reaction [29, 30]. In the presence of luminol, CL arises which

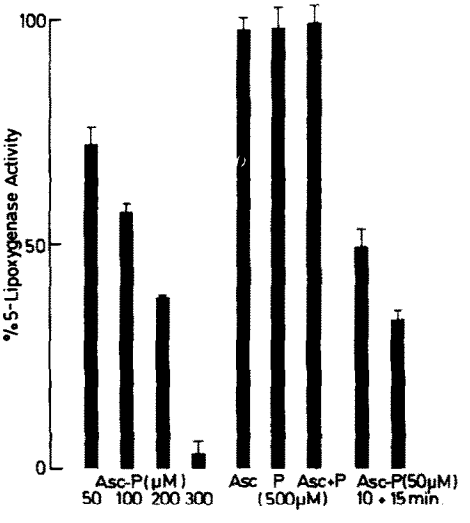

Fig. 9. Effect of ascorbyl palmitate, ascorbate and palmitate on 5-lipoxygenase activity in PMN stimulated by A23187. Preincubation time was 5 min, or 10 or 15 min as indicated. Values are given as means ± SEM from 3 or 4 independent experiments. 100% corresponds to 570 ± 95 ng/10^7 cells (for conditions see Materials and Methods).

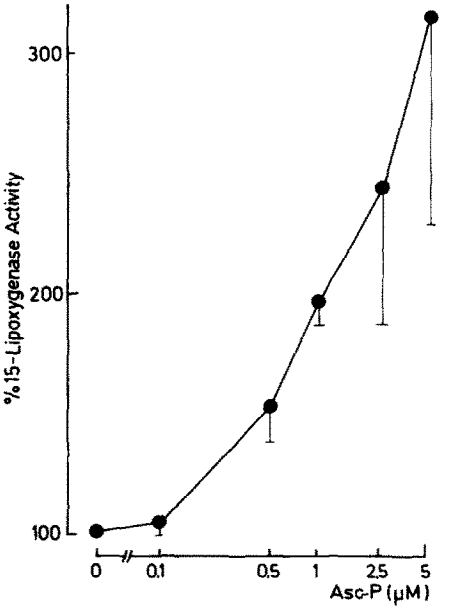

Fig. 11. Effect of ascorbyl palmitate on 15-lipoxygenase activity in PMN homogenates. Values are given as means ± SEM from 4 independent experiments. 100% corresponds to 270 ± 110 ng (for conditions see Materials and Methods).

proceeds in a biphasic time response upon stimulation with FMLP. The two phases can be separated at 25° and were found to respond differently towards antioxidants. Asc only affects efficiently the first rapid phase, whereas its lipophilic derivative quenches the second phase as well. It has been speculated that the first phase could correspond to the action of H_2O_2 and myeloperoxidase in the extracellular compartment, whereas the slower phase could reflect the intracellular space [31, 32]. The latter would be only accessible by lipophilic antioxidants which would be in agreement with our data on the inhibition of the two phases of the luminol-dependent FMLP-stimulated CL. Stimulation by zymosan causes a high degree of phagolysosome formation and this could explain why the difference in inhibition between Asc and Asc-P is more pronounced after cell activation with zymosan than with FMLP.

The CL-enhancer lucigenin seems to react directly with extracellular superoxide radicals without participation of myeloperoxidase [33]. The extracellular origin of the emission would be in agreement with the observed monophasic CL kinetics. In these experiments Asc-P again proved to be more effective than Asc.

Other antioxidants were also active but did not reach the efficiency of Asc-P. The tocopherol derivatives were not inhibitory so proving that the inhibition observed with the other compounds investigated was indeed due to the antioxidant properties. These results may be of physiological significance since the antioxidant potential of Asc may play a role in the intracellular protection of PMN from their own oxidative destruction [34] and also in a scavenging reaction for active oxygen species in the extracellular space.

The effect of Asc on direct PMN-CL emission (in absence of enhancers) elicited by soluble and phagocytic stimuli has been investigated by Kraut *et al.* [35]. Five and 25 mM Asc substantially inhibited the cell response to different stimuli. Anderson *et al.* investigated the effect of Asc on FMLP-stimulated PMN-CL in the presence of luminol [36]. Interestingly, they report an increased intensity of the second peak of the emission in the presence of Asc, whereas the first peak was considerably inhibited. As the experimental conditions used in this report differ from that in our assay system, we attempted to reproduce the reported results using identical conditions. Even so, we were not able to observe the enhancing effect of Asc on the second peak of emission. There are indications, however, that Asc suppresses the self-activation of non-stimulated cells (data not shown). This may explain the data by Andersen *et al.* [36], who reported an increase of the second peak by Asc. By inhibition of the self-activation, Asc prevents the cells from desensitization or self-destruction leading to an enhanced response upon stimulation. We found that low concentrations of BSA efficiently inhibited the self-activation without inhibition of the stimulated response (Baader, unpublished).

The degranulation of lysosomal enzymes can be blocked efficiently by Asc-P but not by Asc or the other antioxidants. Since Asc is a cell constitutent [37], the sensitivity of the secretion process to Asc would be difficult to understand. In addition Asc may not be taken up by neutrophils [38], although contradicting results have been obtained [39].

A mechanism for the inhibition of the various secretory enzymes is lacking at present. One could speculate on a link between oxidative burst and the secretion process. Ozaki *et al.* [15] had reported that several inhibitors of oxygen radical production also impaired degranulation but both effects were not quantitatively correlated, so that a causal link was excluded. Niwa *et al.* [40] even observed an inverse relationship between the release of active oxygen species and secretion.

According to the morphological appearance by electron microscopy, the inhibition of the secretory process was clearly due to a block in degranulation and not to a decrease in enzyme activity. At least for elastase, such a mechanism could have been possible through a blocking by antioxidants of the myeloperoxidase–H_2O_2 mediated inactivation of the α_1-protease inhibitor [41, 42].

Of considerable practical and theoretical importance may be the inhibition of 5-lipoxygenase activity by Asc-P in A23187 stimulated PMN. Again, Asc was without any effect. In homogenates the inhibition by Asc-P was even more pronounced and occurred with a high affinity. In this case a membrane barrier could not be involved since 5-lipoxygenase is a soluble cytosolic enzyme. It is known, however, that lipophilic antioxidants can inhibit the enzyme and constitute, therefore, a potential but not very specific group of 5-lipoxygenase inhibitors [43]. By means of the fatty acid side chain of Asc-P this compound may interact at the lipophilic active site of the enzyme and thus be able to reduce the ferric iron to the inactive ferrous form.

Irrespective of the mechanism of action, the observed efficiency of Asc-P to block three of the main pathophysiological functions of PMN could have therapeutic implications in the development of antiinflammatory drugs.

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