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REVERSIBLE ACTIVATION OF NADPH OXIDASE IN MEMBRANES OF HL-60 HUMAN LEUKEMIC CELLS

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NADPH oxidase in membranes of undifferentiated and dimethylsulphoxide-differentiated HL-60 cells was activated by arachidonic acid (AA) in the presence of Mg^{2+} and a cytosolic cofactor (CF) found in differentiated HL-60 cells. Basal superoxide (O_2^-) formation was enhanced several-fold by addition of the stable GTP-analogue, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), prior to AA and was completely prevented by that of GDP. Basal and GTPγS-stimulated O_2^- formation was terminated by GDP. In the presence of Mg^{2+} or EDTA, basal O_2^- formation ceased after 25 or 10 min, respectively, and was reinitiated by GTPγS or GTPγS plus Mg^{2+}. Albumin terminated O_2^- formation, which was reactivated by AA in the presence of GTPγS. Our results show that (1) activation of NADPH oxidase in HL-60 membranes is dependent on endogenous GTP, Mg^{2+}, AA and CF, which is induced during myeloid differentiation, and that (2) NADPH oxidase activation is a reversible process modulated by exogenous guanine nucleotides at various stages of activity of NADPH oxidase. We suggest crucial roles of guanine nucleotide-binding proteins in the activation, deactivation and reactivation of the enzyme.

Human neutrophils contain a plasma membrane-bound NADPH oxidase, which catalyzes O_2^- formation and can be activated by the chemotactic peptide, FMLP (1). In addition, FMLP activates phospholipase C-catalyzed polyphosphoinositide degradation and phospholipase A_2-mediated release of AA (2,3). Both effects are assumed to be mediated by guanine nucleotide-binding proteins (2,3).

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Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; CB, cytochalasin B; CF, cytosolic cofactor; DMSO, dimethylsulphoxide; FMLP, N-formyl-L-methionyl-L- leucyl-L-phenylalanine; GDPβS, guanosine 5'-O-(2-thiodiphosphate); GTPγS, guanosine 5'-O-(3-thiotriphosphate); G-protein, guanine nucleotide-binding protein; O_2^-, superoxide anion; PMA, 4β-phorbol 12-myristate 13-acetate.

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FMLP-induced activation of NADPH oxidase has been suggested to be mediated by protein kinase C (4), but there exists recent evidence that the FMLP-induced activation of O_2^- formation is independent of protein kinase C (5,6). As AA induces O_2^- formation in intact neutrophils (7,8) and in cell-free systems (9-14), AA may serve as an intracellular messenger for activation of NADPH oxidase. Activation of the enzyme in cell-free systems depends on Mg^{2+} and a neutrophil-specific cytosolic cofactor, which is apparently different from protein kinase C (11-14). Recent results indicate that a G-protein is involved in the regulation of NADPH oxidase by FMLP (10-12). In intact neutrophils, NADPH oxidase activation by FMLP and AA is a reversible process, but the mechanisms underlying deactivation of the enzyme are not known (8,15). Once activated, NADPH oxidase in neutrophil membranes cannot be deactivated, indicating that a mechanism for termination of O_2^- production present in intact cells is lost during cell disruption (12-14). In our present study, we show reversible activation of NADPH oxidase in membranes of HL-60 human leukemic cells.

MATERIALS AND METHODS

PMA, FMLP, CB, superoxide dismutase, ferricytochrome C (Type III) and AA (grade I, 99% pure) were obtained from Sigma Chemie (Taufkirchen, F.R.G.). A stock solution of 16.4 mM AA in 100% ethanol was prepared under nitrogen and stored at -20°C. All nucleotides were from Boehringer Mannheim (Mannheim, F.R.G.). DMSO, BSA (0.0015% fatty acids) and type 20/32 Visking dialysis tubings (exclusion limit of 8 to 15 kDa) were from Serva (Heidelberg, F.R.G.). All cell culture media were obtained from Biochrom (Berlin, F.R.G.).

HL-60 cells were grown in RPMI-1640 culture medium supplemented with 20% (v/v) fetal calf serum, 1% (v/v) non-essential amino acids, 2 mM L-glutamine, 50 U/ml penicillin and 50 µg/ml streptomycin in a humidified atmosphere with 7% CO_2. To induce myeloid differentiation, HL-60 cells were seeded at a density of 10^6 cells/ml and cultured for 6 days in the medium supplemented with 1.25% (v/v) DMSO (16). Panoptic Pappenheim stains revealed that undifferentiated HL-60 cells consisted of > 95% promyelocytes. Differentiated HL-60 cells consisted of > 70% metamyelocytes or cells beyond this stage. Undifferentiated, intact HL-60 cells did not generate O_2^- upon stimulation with FMLP (1 µM) plus CB (1 µg/ml) or AA (164 µM) and generated 6 nmoles of $O_2^-/10^7$ cells upon stimulation with PMA (100 ng/ml); differentiated HL-60 cells generated 29, 31 and 73 nmoles of $O_2^-/10^7$ cells, respectively, upon exposure to these stimuli.

HL-60 cells were disintegrated by nitrogen cavitation in a buffer consisting of 50 mM KH_2PO_4, 100 mM NaCl and 0.5 mM EDTA, pH 7.0. EDTA and β-mercaptoethanol were added to broken cells to give final concentrations of 3 and 15 mM, respectively. Nuclei were removed by centrifugation for 1 min at 500 x g. The postnuclear supernatant fluid was centrifuged for 15 min at 30,000 x g. The pellet fraction was termed "membranes" and resuspended in 10 mM triethanolamine/HCl, pH 7.0. The supernatant fraction was re-centrifuged for 1 h at 180,000 x g. The subsequent supernatant fraction was termed "cytosol" and contained CF activity. Cytosol was dialyzed extensively against a buffer composed

of 50 mM KH_2PO_4, 100 mM KCl and 1 mM EDTA, pH 7.0, to remove low molecular weight compounds including Ca^{2+}, Mg^{2+}, ß-mercaptoethanol and ATP as described (11). Membranes and cytosol were stored at -80°C for 6 months without loss of activity. Protein determination was performed according to Lowry et al. (17).

NADPH oxidase activity of HL-60 membranes was monitored by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase at 25°C (10). Unless stated otherwise, assay mixtures (500 µl) contained 38 µg of membrane protein and 200 µg of cytosolic protein from differentiated HL-60 cells, 500 µM NADPH, 10 µM FAD, 100 µM ferricytochrome C, $MgCl_2$ (2 mM free Mg^{2+}) and 50 mM triethanolamine/HCl, pH 7.0. Ethanol up to 2% (v/v) was without inhibitory effect on O_2^- formation. The results shown in Figs. 1 to 3 are representative for at least three independent experiments.

RESULTS AND DISCUSSION

AA (200 µM) activated O_2^- formation in membranes of undifferentiated and DMSO-differentiated HL-60 cells in the presence of Mg^{2+} and CF from differentiated HL-60 cells (Table 1). Addition of the stable GTP analogue, GTPγS, prior to AA enhanced the rate of O_2^- generation several-fold. These results indicate that all components of NADPH oxidase and the G-proteins are present both in undifferentiated and differentiated HL-60 cells. The specific activity of NADPH oxidase in undifferentiated HL-60 membranes was half of that found in differentiated HL-60 membranes and may be due to a lower content of cytochrome b_{-245} in the undifferentiated cells (18). CF activity from undifferentiated

Table 1. Differentiation-dependent expression of NADPH oxidase activity in membranes of HL-60 human leukemic cells

Source of membranes	Addition	NADPH oxidase activity (nmol x mg^{-1} x min^{-1})	
		Source of cytosolic cofactor	
		undifferentiated HL-60 cells	DMSO-differentiated HL-60 cells
undifferentiated HL-60 cells	none	2 ± 1	14 ± 3
	GTPγS (10 µM)	13 ± 3	47 ± 6
DMSO-differentiated HL-60 cells	none	4 ± 1	31 ± 5
	GTPγS (10 µM)	18 ± 4	72 ± 10

Reaction mixtures contained 38 µg of membrane protein and 200 µg of cytosolic protein from undifferentiated or DMSO-differentiated HL-60 cells. GTPγS or solvent was added to the assay mixtures 2 min prior to AA (200 µM). Data represent the mean ± SEM of three experiments.

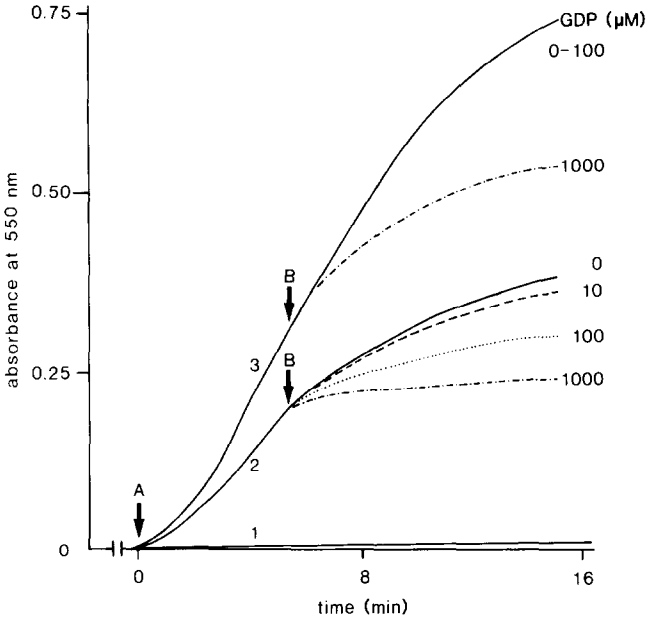

Fig. 1. Deactivation of O_2^- formation in HL-60 membranes by GDP. Two minutes prior to AA (200 μM, arrow A), GDP (1 mM, trace 1), solvent (trace 2) or GTPγS (10 μM, trace 3) were added to reaction mixtures. Arrow B marks the addition of GDP at the concentrations indicated.

HL-60 cells amounted to less than 30% of that of differentiated cells, indicating that the expression of CF was closely related to myeloid differentiation (19). Similar to undifferentiated HL-60 cells, neutrophils from certain patients with chronic granulomatous disease were found to be devoid of CF (14). Each cell type possesses a significant protein kinase C activity (14,20), confirming the view that CF is different from protein kinase C (11-14).

GDP (1 mM) completely prevented basal O_2^- formation and concentration-dependently terminated basal and GTPγS-stimulated enzyme activity (Fig. 1). The inhibitory effect of GDP on basal O_2^- formation was half-maximal at a concentration of 100 μM. In the presence of GTPγS, GDP was inhibitory only at high concentrations (1 mM). Similar effects as with GDP were seen with the stable GDP analogue, GDPβS, whereas ADP and UDP were inactive (data not shown). These results indicate that GDP competes with G-protein-activating nucleotides, i.e. with endogenous GTP and less effectively with exogenous GTPγS, thus promoting inactivation of G-proteins and consequent deactivation

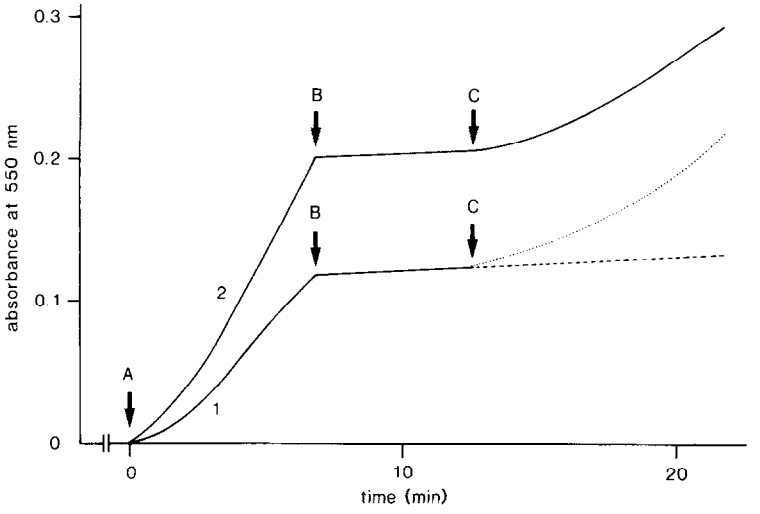

Fig. 2. Deactivation of O_2^- formation in HL-60 membranes by BSA and its reactivation by AA and GTPγS. Two minutes prior to AA (200 μM, arrow A), solvent (trace 1) or GTPγS (10 μM, trace 2) were added to assay mixtures. Arrow B indicates the addition of BSA (33.3 μM), arrow C the re-addition of 200 μM AA (traces 1 and 2 [---]) or 200 μM AA plus 10 μM GTPγS (trace 2 [···]).

of NADPH oxidase. The results also implicate that the induction of basal O_2^- generation rates does not only require AA and CF but also active G-proteins. In contrast to the findings in HL-60 membranes, GDP and GDPβS, at concentrations up to 5 mM, only partially prevented basal O_2^- formation in neutrophil membranes and were without effect when added after AA (14).

In neutrophil membranes, NADPH oxidase activation by AA was reported to be irreversible, because removal of AA by BSA or of AA and CF by centrifugation did not substantially affect O_2^- formation (12-14). In HL-60 membranes BSA at the concentration employed (33.3 μM) binding 200 μM AA (8), rapidly terminated basal and GTPγS-stimulated O_2^- formation induced by 200 μM AA (Fig. 2). Re-addition of AA failed to re-induce O_2^- formation unless GTPγS was present. These results indicate that O_2^- formation in HL-60 membranes depends on the presence of AA and is reversed upon removal of AA. They further show that AA is necessary but not sufficient for the reactivation of O_2^- formation, which requires the additional presence of a stimulatory guanine nucleotide.

In the presence of $MgCl_2$ (2 mM free Mg^{2+}), basal O_2^- formation ceased after 25 min and led to the generation of 0.40 μmoles of O_2^-/mg protein corre-

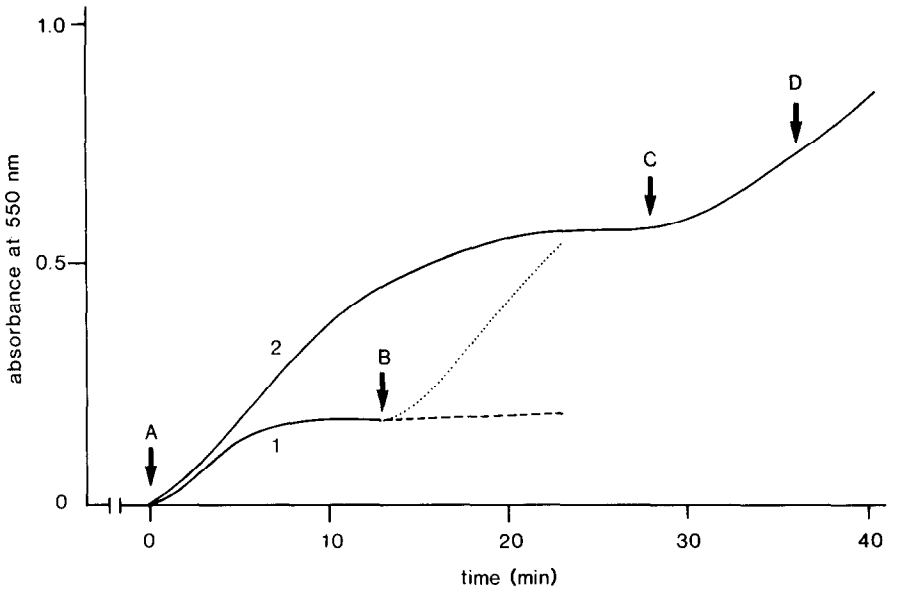

Fig. 3. Effects of Mg^{2+} and EDTA on time course of O_2^- formation in HL-60 membranes and reactivation of O_2^- formation by Mg^{2+} and GTPγS. Two minutes prior to AA (200 μM, arrow A), 5 mM EDTA (trace 1) or $MgCl_2$ (2 mM free Mg^{2+}) (trace 2) were added to assay mixtures. Arrow B indicates the addition of 10 μM GTPγS [---] or 10 μM GTPγS plus $MgCl_2$ (2 mM free Mg^{2+}) [···]. Arrow C marks the addition of 10 μM GTPγS, arrow D that of 10 mM EDTA.

sponding to 39.5 nmoles of $O_2^-/10^7$ cells (Fig. 3). Similar rates were obtained with intact differentiated HL-60 cells upon stimulation with AA, PMA or FMLP plus CB (see MATERIALS AND METHODS). In the presence of 5 mM EDTA, O_2^- formation ceased after 10 min and led to the generation of 0.13 μmoles of O_2^- /mg protein. O_2^- formation was re-initiated by GTPγS when $MgCl_2$ (2 mM free Mg^{2+}) was present. As the γ-phosphoryl group of GTP and GTPγS is possibly bound in the nucleotide pocket of G-proteins via Mg^{2+} (21), these results underline the importance of G-proteins in the activation and reactivation of O_2^- formation. EDTA (10 mM), added after GTPγS, did not inhibit O_2^- formation, indicating that Mg^{2+} is required only for the initiation of O_2^- formation or that Mg^{2+} activation is only slowly reversed by EDTA (22).

In summary, we provide evidence that G-proteins are not only involved in the activation but also in the deactivation and reactivation of NADPH oxidase in HL-60 membranes. The reasons for the observed differences in NADPH oxidase regulation between HL-60 and neutrophil membranes (12-14) are not yet known,

but the HL-60 membranes may provide a useful system to further elucidate the regulation of the enzyme.

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