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DIFFERENTIAL EXPRESSION OF CYTOSOLIC ACTIVATION FACTORS FOR NADPH OXIDASE IN HL-60 LEUKEMIC CELLS

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Activation of NADPH oxidase in undifferentiated HL-60 leukemic cells and in HL-60 cells differentiated along the myeloid pathway with dibutyryl cyclic AMP (dbcAMP) or dimethyl sulfoxide (Me_2SO) was studied. Upon stimulation with a calcium ionophore, a phorbol ester, arachidonic acid or γ-hexachlorocyclohexane, Me_2SO-differentiated HL-60 cells generated superoxide (O_2^-) at higher rates than dbcAMP-differentiated cells. Undifferentiated cells generated O_2^- only at low rates upon stimulation with the above agents. In cell-free systems, NADPH oxidase activity was reconstituted by combining membranes of undifferentiated or dbcAMP- or Me_2SO-differentiated HL-60 cells, cytosol of Me_2SO-differentiated cells and arachidonic acid. This basal O_2^- formation was enhanced several-fold by guanosine 5'-O-(3-thiotriphosphate) (GTP[γS]), a potent activator of guanine nucleotide-binding proteins. In contrast, cytosol of dbcAMP-differentiated cells reconstituted O_2^- formation only in the presence of GTP[γS], and cytosol of undifferentiated cells was inactive. Submaximally stimulatory amounts of cytosolic protein of Me_2SO- and dbcAMP-differentiated cells synergistically stimulated O_2^- formation in the presence but not in the absence of GTP[γS]. We conclude that differentiations of HL-60 cells with Me_2SO and dbcAMP are not equivalent with respect to activation of NADPH oxidase and that two cytosolic activation factors are involved in the regulation of this effector system.

Human neutrophils and HL-60 leukemic cells possess an NADPH oxidase which catalyzes O_2^- formation and is activated by the chemotactic peptide, fMet-Leu-Phe. Chemotactic peptides activate phospholipases C and A_2 *via* G proteins [1-3], leading to the release of diacylglycerol and arachidonic acid.

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ABBREVIATIONS
dbcAMP, dibutyryl cyclic AMP; G protein, guanine nucleotide-binding protein; GTP[γS], guanosine 5'-O-(3-thiotriphosphate); fMet-Leu-Phe, *N*-formyl-*L*-methionyl-*L*-leucyl-*L*-phenylalanine; Me_2SO, dimethyl sulfoxide; NBT, nitroblue tetrazolium; O_2^-, superoxide anion; PMA, phorbol myristate acetate.

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Activation of NADPH oxidase by fMet-Leu-Phe has been suggested to involve protein kinase C, calcium mobilization and more direct regulation by G proteins [3]. PMA activates NADPH oxidase *via* protein kinase C, and activation of O_2^- formation by the calcium ionophore, A 23187, involves various calcium-dependent processes [3]. Arachidonic acid may activate NADPH oxidase by changing the lipid environment of the enzyme or by stimulating protein kinase C [3,4]. In addition, the insectizide, γ-hexachlorocyclohexane, has been suggested to stimulate O_2^- formation by activating phospholipase C [5].

In cell-free systems, NADPH oxidase activity is reconstituted by combining membranes and cytosol of neutrophils and fatty acids or SDS [6-17]. Fatty acid-induced O_2^- formation is enhanced several-fold by the stable GTP analogue, GTP[γS] [6-11], suggesting the involvement of a G protein in the regulation of NADPH oxidase [18]. Cytosols of neutrophils, macrophages and HL-60 cells differentiated with Me_2SO or vitamin D_3 contain a cytosolic activation factor for NADPH oxidase [6-17]. In contrast, neutrophil cytosols of patients with an autosomal recessive form of chronic granulomatous disease and cytosols of undifferentiated HL-60 cells are devoid of this factor [8,16,19]. The cytosolic activation factor is different from protein kinase C or a lipoxygenase but its identity is still unknown [7-11,17].

Myeloid differentiation of HL-60 cells is induced by a variety of compounds such as Me_2SO and dbcAMP. The differentiation programs induced by these agents are not equivalent with respect to expression of formyl peptide receptors [20,21]. All these findings prompted us to study the differentiation-dependent activation of NADPH oxidase in HL-60 cells. We report here on differential activation of NADPH oxidase in HL-60 cells differentiated with dbcAMP and Me_2SO. Evidence is presented that two cytosolic activation factors are involved in the regulation of NADPH oxidase.

MATERIALS AND METHODS

γ-Hexachlorocyclohexane, A 23187 and NBT were obtained from Sigma Chemie (Deisenhofen, FRG). A 23187 (10 mM) and γ-hexachlorocyclohexane (50 mM) were dissolved in Me_2SO. Reagents for measurement of O_2^- generation and cell culture media have been described elsewhere [6-9,22].

HL-60 cells were cultured in suspension culture as described recently [8,9,22]. To induce differentiation, HL-60 cells were seeded at a density of 10^6 cells/ml and were cultured for 48 h with 0.2 mM dbcAMP or for 120 h with 160 mM Me_2SO [8,9,20-22]. Cell viability after differentiation was 90-95% as revealed by trypan blue dye exclusion. Undifferentiated HL-60 cells consisted of 89% promyelocytes, 10% myelocytes and 1% myelocytes, 8% of the cells being NBT-positive following stimulation with PMA. DbcAMP-differentiated cells consisted of 2% promyelocytes, 54% myelocytes, 38% metamyelocytes and 6% neutrophils, 84% of the cells being NBT-positive. Me_2SO-differentiated cells consisted of 1% promyelocytes, 56% myelocytes, 26% metamyelocytes and 17% neutrophils, 96% of the cells being NBT-positive. HL-60 membranes and cytosol were prepared as described [8]. Protein determination was performed according to Lowry et al. [23], using bovine serum albumin as standard.

NADPH oxidase-catalyzed O_2^- formation was monitored by continuous measurement of ferricytochrome C reduction inhibitable by superoxide dismutase, using an Uvikon 810 dual beam spectrophotometer (Kontron, Eching, FRG) [6-9,22]. Reference cuvettes contained the components listed below plus 50 μg of superoxide dismutase. Reaction mixtures for determination of O_2^- formation in the cell-free system (500 μl) contained 50 μg of membrane protein, various amounts of cytosolic protein, 10 μM FAD, 500 μM NADPH, 100 μM ferricytochrome C, 2 mM $MgCl_2$, 20 mM KH_2PO_4, 40 mM KCl and 20 mM triethanolamine/HCl, pH 7.0. Reaction mixtures were preincubated for 2 min at 25°C. O_2^- formation was initiated by the addition of arachidonic acid (200 μM). Assay mixtures for measurement of O_2^- formation in intact cells (1 ml) contained 5 x 10^6 HL-60 cells, 100 uM ferricytochrome C and a buffer consisting of (mM) 138 NaCl, 6 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5.5 glucose and 20 Hepes, pH 7.4. Cells were incubated for 3 min at 37°C prior to addition of the stimuli. V_{max} of O_2^- formation was calculated from the linear parts of absorbance curves.

RESULTS

Activation of NADPH oxidase was studied in intact HL-60 cells. In preliminary experiments, the concentrations of stimuli given in Table 1 were found to be maximally effective to activate O_2^- formation (data not shown). In

Table 1: Differentiation-dependent activation of NADPH oxidase in intact HL-60 cells

Stimulus	O_2^- generation (nmol x 10^7 cells^{-1} x min^{-1})		
	differentiation-inducing agent		
	none	dbcAMP	Me_2SO
None	0	0	0
PMA (100 ng/ml)	0.5 ± 0.3	14.8 ± 3.5	50.5 ± 5.4
A 23187 (50 μM)	0.7 ± 0.5	0.9 ± 0.1	4.8 ± 0.4
AA (160 μM)	0.6 ± 0.5	10.3 ± 0.9	29.7 ± 4.8
HCCH (500 μM)	0	15.3 ± 1.8	32.1 ± 2.1
fMet-Leu-Phe (1 μM)	1.7 ± 0.4	20.9 ± 4.5	10.2 ± 0.7

Arachidonic acid (AA), γ-hexachlorocyclohexane (HCCH). Cells stimulated with fMet-Leu-Phe were pretreated with cytochalasin B (1 μg/ml) for 3 min. Data shown represent the mean ± SD of eight experiments performed with different cell preparations.

Table 2: Differentiation-dependent activation of NADPH oxidase in cell-free systems of HL-60 cells

Differentiation-inducing agent	NADPH oxidase activity (nmol x mg^{-1} x min^{-1})	
	addition	
	none	GTP[γS] (1 μM)
none	0.5 ± 0.1	1.1 ± 1.0
dbcAMP	0.7 ± 0.5	10.5 ± 1.0
Me_2SO	10.1 ± 1.1	31.5 ± 2.3

Assays contained 50 μg of membrane protein and 125 μg of cytosolic protein. GTP[γS] or solvent (control) was added to assay mixtures 2 min prior to arachidonic acid (200 μM). Data shown represent the mean ± SEM of three experiments performed with one preparation of HL-60 cells. Experiments with three different preparations of HL-60 cells gave similar results.

the absence of stimuli, HL-60 cells did not generate O_2^-. Upon exposure to PMA, arachidonic acid, γ-hexachlorocyclohexane or fMet-Leu-Phe plus cytochalasin B, dbcAMP-differentiated cells generated O_2^- at rates at least 12-fold higher than those of undifferentiated cells. In Me_2SO-differentiated cells, PMA, A 23187, arachidonic acid and γ-hexachlorocyclohexane induced O_2^- generation at rates up to 5-fold higher than those observed in dbcAMP-differentiated cells. In contrast, Me_2SO-differentiated cells generated O_2^- at rates half of those of dbcAMP-differentiated cells upon exposure to fMet-Leu-Phe plus cytochalasin B, possibly due to the expression of lower numbers of formyl peptide receptors in the latter cells [21].

Activation of NADPH oxidase was studied in cell-free systems (Table 2). Basal, arachidonic acid-induced O_2^- formation in cell-free systems of undifferentiated and dbcAMP-differentiated HL-60 cells was very low in comparison to that of Me_2SO-differentiated cells. GTP[γS] substantially enhanced O_2^- formation in cell-free systems of Me_2SO- and dbcAMP-differentiated cells but not in cell-free systems of undifferentiated HL-60 cells. Trypsin-treated cytosols of dbcAMP- and Me_2SO-differentiated cells did not reconstitute NADPH oxidase activity (data not shown), indicating that trypsin-sensitive proteins were involved in reconstitution of O_2^- formation. Cross-mixing experiments with membranes of undifferentiated or dbcAMP-differentiated cells and cytosol of

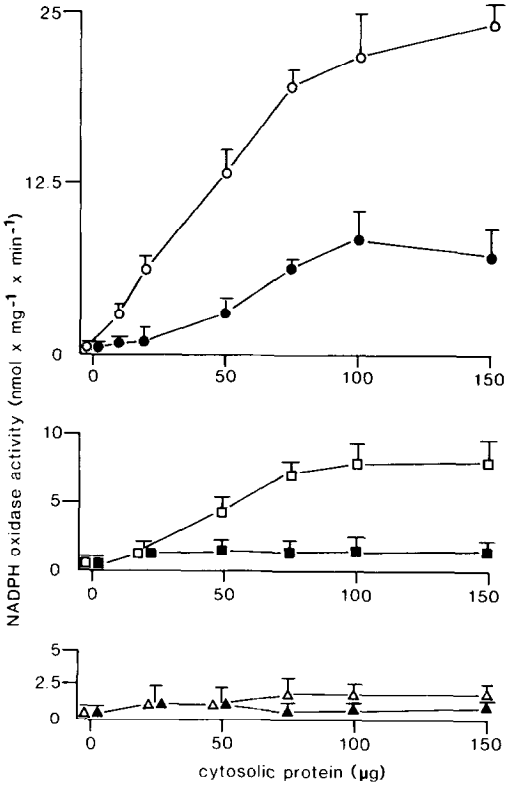

Fig. 1: Differentiation-dependent expression of cytosolic activation factors for NADPH oxidase in HL-60 cells. NADPH oxidase activity was measured in the absence (closed symbols) or presence (open symbols) of GTP[γS] (1 μM) and different amounts of cytosolic protein (0-150 μg of protein/assay). Assays contained 50 μg of membrane protein of undifferentiated HL-60 cells and cytosol of Me_2SO-differentiated HL-60 cells (o, •), cytosol of dbcAMP-differentiated cells (□, ■) or cytosol of undifferentiated cells (△, ▲). Data shown represent the mean ± SD of three to five experiments performed with one preparation of HL-60 cells. Experiments with three different preparations of HL-60 cells gave similar results.

Me_2SO-differentiated cells revealed that their specific activities amounted to 60-95% of those of Me_2SO-differentiated cells (data not shown), indicating that the membrane component of NADPH oxidase was not defective in HL-60 cells.

The above findings suggested that differentiation of HL-60 cells was associated with changes in the activity of cytosolic activation factors for NADPH oxidase. In order to prevent interference with residual membrane-bound cytosolic activation factors, in all further experiments membranes of undifferentiated HL-60 cells were used. Reconstitution of NADPH oxidase activity was studied as a function of the amount of cytosolic protein added (Fig. 1). Cytosol of undifferentiated HL-60 cells did not reconstitute basal and

GTP[γS]-stimulated O_2^- formation. In contrast, cytosolic protein of Me_2SO-differentiated cells concentration-dependently reconstituted basal and GTP[γS]-stimulated O_2^- formation, reaching saturation at a ratio of cytosolic protein to membrane protein of 3. In addition, GTP[γS] shifted the concentration response function to cytosolic protein of Me_2SO-differentiated cells to the left. Cytosolic protein of dbcAMP-differentiated cells concentration-dependently reconstituted GTP[γS]-stimulated but not basal O_2^- generation. Reconstitution of GTP[γS]-stimulated O_2^- formation by cytosol of dbcAMP-differentiated cells required higher amounts of cytosolic protein than the one with cytosolic protein of Me_2SO-differentiated cells. In addition, the absolute stimulatory effects of GTP[γS] with cytosol of dbcAMP-differentiated were smaller than those with Me_2SO-differentiated cells.

Cross-mixing experiments with cytosols of HL-60 cells were performed. A three-fold excess of cytosolic protein of undifferentiated cells in relation to cytosolic protein of dbcAMP- or Me_2SO-differentiated cells did not inhibit O_2^- formation (data not shown), suggesting that cytosol of undifferentiated cells did not contain inhibitory factors. Cytosolic protein of dbcAMP-differentiated cells did not enhance basal NADPH oxidase activity reconstituted by submaximally and maximally stimulatory amounts of cytosolic protein of Me_2SO-differentiated cells (Fig. 2). The combination of submaximally stimulatory amounts of cytosolic protein of Me_2SO-differentiated cells and of dbcAMP-differentiated cells synergistically enhanced GTP[γS]-stimulated O_2^- formation (see Fig. 2). With cytosolic proteins of Me_2SO- and dbcAMP-differentiated cells being present at maximally stimulatory amounts, no synergism was observed, indicating that both cytosols acted by a mechanism they had in common.

DISCUSSION

Our results suggest that two cytosolic activation factors are involved in the regulation of O_2^- formation. The first factor reconstitutes basal, arachidonic acid-induced O_2^- formation and is present in Me_2SO-differentiated HL-60 cells but not in dbcAMP-differentiated and undifferentiated cells. Basal,

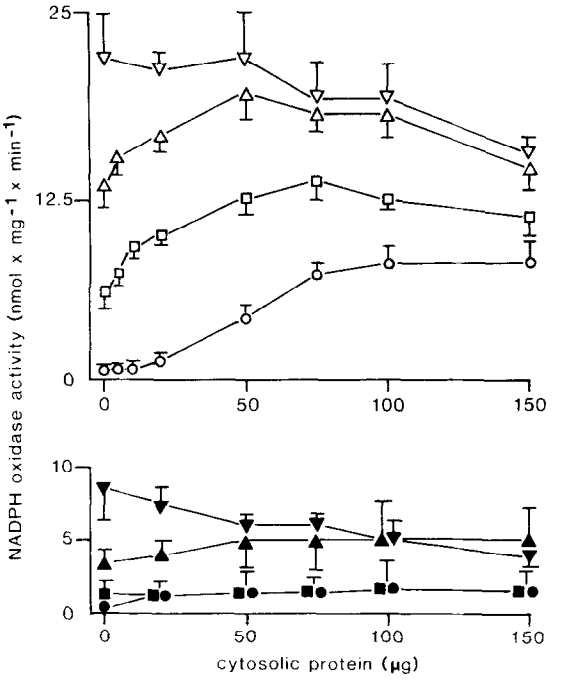

Fig. 2: Interaction of cytosolic activation factors for NADPH oxidase of dibutyryl cyclic AMP-differentiated HL-60 cells with those of dimethyl sulfoxide-differentiated cells. NADPH oxidase activity was determined in the absence (closed symbols) or presence (open symbols) of GTP[γS] (1 µM) and different amounts of cytosolic protein of dbcAMP-differentiated cells (0-150 µg of protein/assay). Reaction mixtures contained 50 µg of membrane protein of undifferentiated cells and three fixed amounts of cytosolic protein of Me_2SO-differentiated cells: 0 µg (o, •), 20 µg (□, ■), 50 µg (△, ▲), 100 µg (▽, ▼). O_2^- formation was initiated by the addition of arachidonic acid. Data shown represent the mean ± SD of three to five experiments performed with one preparation of HL-60 cells. Similar results were obtained with three different cell preparations.

arachidonic acid-induced O_2^- formation is also reconstituted by cytosol of macrophages, neutrophils, HL-60 cells differentiated with vitamin D_3 [6-17,19, 20] and HL-60 cells differentiated with retinoic acid (unpublished results). The second factor mediates G protein regulation of NADPH oxidase in cell-free systems. This factor is present in dbcAMP- and Me_2SO-differentiated cells, in neutrophils [6-10] and in HL-60 cells differentiated with vitamin D_3 or retinoic acid (unpublished results) but not in cytosol of undifferentiated HL-60 cells. Each factor *per se* is sufficient to activate NADPH oxidase in the absence or presence of GTP[γS]. In addition, both factors interact synergistically to reconstitute O_2^- formation in the presence of GTP[γS]. This interpretation is supported by the finding that the absolute stimulatory effects of

GTP[γS] were substantially greater with cytosol of Me_2SO-differentiated cells than with cytosol of dbcAMP-differentiated cells. In addition, lower amounts of cytosolic protein of Me_2SO-differentiated cells than of dbcAMP-differentiated cells were required to reconstitute GTP[γS]-stimulated O_2^- formation. Furthermore, cytosols of dbcAMP-and Me_2SO- differentiated HL-60 cells synergistically reconstituted GTP[γS]-stimulated O_2^- formation.

In contrast to differentiated HL-60 cells, undifferentiated HL-60 cells generated O_2^- at very low rates upon exposure to PMA, A 23187, arachidonic acid or γ-hexachlorocyclohexane. The mechanisms by which these agents activate NADPH oxidase may involve activation of protein kinase C, calcium mobilization, interaction with the membrane component of NADPH oxidase and stimulation of phospholipase C [3-5]. Undifferentiated cells possess substantial activities of the membrane component of NADPH oxidase, phospolipase C [24] and protein kinase C [25]. In cell-free systems, cytosol of undifferentiated cells did neither reconstitute basal nor GTP[γS]-stimulated enzyme activity. These data suggest that cytosolic activation factors for NADPH oxidase are also involved in the activation of O_2^- formation in intact HL-60 cells by A 23187, PMA, arachidonic acid and γ-hexachlorocyclohexane. However, dbcAMP-differentiated HL-60 cells possess only the cytosolic activation factor mediating G protein regulation of NADPH oxidase but generate O_2^- at substantial rates upon stimulation with PMA, γ-hexachlorcyclohexane or arachidonic acid which circumvent activation of G proteins. Therefore, the precise role of cytosolic activation factors in the activation of NADPH oxidase in intact cells remains to be established.

Intact neutrophils of patients with an autosomal recessive form of chronic granulomatous disease do not generate O_2^- upon stimulation with various stimuli [15,19]. In addition, neutrophil cytosol of these patients fails to reconstitute O_2^- formation in cell-free systems [19]. Similarily to undifferentiated HL-60 cells, the defect of the cytosolic activation factor in chronic granulomatous disease is not due to the presence of inhibitory components. Intact dbcAMP-differentiated HL-60 cells generated O_2^- at substantial

rates upon stimulation with various agents but failed to reconstitute basal O_2^- formation in cell-free systems. This type of defect in dbcAMP-differentiated HL-60 cells has no known equivalent in pathology of human neutrophils. In conclusion, the analysis of differentiation-dependent activation of NADPH oxidase in HL-60 cells provides a promising approach to analyze the regulation of this effector system.

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