Vol. 177, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 31, 1991 Pages 504-510

DIALDEHYDE-GDP BLOCKS ACTIVITY OF CYTOSOLIC COMPONENTS OF NEUTROPHIL NADPH OXIDASE

Eti Klinger and Irit Aviram

The Department of Biochemistry, Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv 69978, Israel

Received April 26, 1991

Superoxide production by neutrophil NADPH oxidase activated in a cell-free system consisting of plasma membranes, cytosol and arachidonate is enhanced by nonhydrolyzable analogs of GTP and reduced by GDP. To characterize the interaction of guanine nucleotides with the system, dialdehyde analogs of GTP and GDP (oGTP and oGDP) were employed. oGDP or oGTP caused an irreversible and dose dependent inactivation of NADPH oxidase-supporting cytosolic activity. Cytosol was fractionated on S and Q Sepharose ion exchange columns into three fractions, combinations of which synergistically supported activation of NADPH oxidase. Two fractions shown by immunoblotting to contain the oxidase-linked p47 and p67 proteins were inactivated by oGDP. Labeling with [α-^{32}P]-oGTP lead to incorporation of the label into several proteins.

NADPH-dependent superoxide generation can be induced by exposure of isolated neutrophil membranes and cytosolic components to anionic detergents (fatty acids e.g. arachidonate or SDS) (1,2) . This artificially elicited activity resembles the physiological action of membrane-bound NADPH oxidase of stimulated phagocytes (1,2). Among other similarities, oxidase activity in the cell-free system can be enhanced by poorly hydrolyzable guanosine triphosphate analogs (GTPγS or GppNHp) or fluoride and inhibited by GDP or GDPβS (3-6). These findings implicated guanine nucleotide binding proteins in the oxidase activation process. Their location (cytosolic or membrane-associated), nature and relation to the catalytic complex of NADPH oxidase remained unknown.

The complete functional NADPH oxidase complex has not yet been isolated. It is believed to consist of a dimeric, membrane-bound cytochrome b558, an NADPH-binding flavoprotein and several cytosolic components including the two recently characterized 47 kDa and 67 kDa proteins (1,2,7-9).

Techniques of chemical modifications and affinity labeling have been helpful in the identification and characterization of various cellular activities. Periodate-oxidized nucleotides in which the

0006-291X/91 $1.50

ribose ring has been cleaved to give 2'3'-dialdehyde analogs , have been successfully employed in studies of nucleotide-utilizing proteins (10-13). If binding of the analog involves formation of a Schiff base between its aldehyde group and a properly oriented amino group on the enzyme , reduction of the complex with [^{3}H]- sodium borohydride stabilizes the bond and labels the binding protein (10,11). In other cases covalent binding of oxidized nucleotides involves formation of a dihydroxymorpholino compound between the analog and a lysyl side chain on the protein (12,13).

In this report, we describe our attempts to characterize the guanine nucleotide-binding site/s responsible for modulation of neutrophil NADPH oxidase in a cell-free system, employing the 2'3'-dialdehyde GDP or GTP (oGDP or oGTP) .

Materials and Methods

Fractionation of neutrophils: Human neutrophils were isolated from fresh buffy coats by standard procedures. Cells were disrupted by sonication and fractionated into soluble cytosolic and light membrane fractions as described (14) .

NADPH oxidase activation and assay: Arachidonate-dependent activation was performed in two steps essentially as described (14). In the first step, membranes and cytosol (1-2x10^6 and 2-4x10^6 cell eq respectively) were incubated with arachidonate (150-300μM) in 0.1-0.2 ml of 0.34M sucrose/50 mM potassium phosphate pH 7.0 buffered saline (sucrose-PBS) containing 1 mM EGTA and 5 mM $MgCl_2$. After 6 minutes at 30^{o}C, 0.5 ml sucrose-PBS , 0.2 mM NADPH and 80μg cytochrome c were added and reduction of cytochrome c was followed (14) .

Ion exchange fractionation of cytosol: Fractionation was performed using a modified by us procedure of Pilloud et al.(15) . One-two milliliters of cytosol were loaded on an S Sepharose (Sigma) column (1.0 ml) equilibrated with 20 mM pH 7.5 Hepes - 0.15 M NaCl buffer (Hepes-NaCl). The column was washed with 5 ml of the equilibration buffer and a 0.15-0.5M NaCl gradient (total volume of 10ml) was applied. In some experiments elution was carried out by 0.35M NaCl (S350). Fractions of 1 ml were collected. Fractions 2-4 of the flowthrough of the S Sepharose column (S150) were transferred to a (1.0 ml) Q Sepharose (Sigma) column equilibrated with Hepes - NaCl and washed with 5 ml of the same buffer (Q150). Proteins were eluted by 5 ml of Hepes-0.3 M NaCl (Q300) followed by 5 ml of Hepes-0.5 M NaCl (Q500). Column fractions (30-50μl) were assayed for their ability to support cell-free activation of NADPH oxidase in the presence of a saturating concentration of GTPγS (7μM) .

Irreversible inhibition by oGDP: Cytosol or membranes (0.2-0.25 ml) were incubated with 0.1mM-1.0mM oGDP for one hour at room temperature and separated from unbound reagent on a Sephadex G-25 column (0.5x20 cm) equilibrated with 10 mM Hepes pH 6.8/0.13M NaCl/0.5mM $MgCl_2$. Inhibition by oGDP of the S and Q Sepharose fractions was determined by one hour incubation with 0.1 mM oGDP and assay, omitting the gel filtration step . All experiments were performed in duplicates and repeated at least three times.

Labeling of oGTP-binding proteins with dialdehyde [α-^{32}P]-GTP. 1 μCi of [α-^{32}P]-GTP (Amersham,3000 Ci/mmol) was added to cytosol and column fractions (50μl) followed by 20 nmol of sodium periodate and incubated one hour in dark at room temperature. The reaction was quenched by 20 nmoles of ethylene glycol (10) and the fractions subjected to SDS-PAGE in 5-15% gradient gels. Gels were dried in vacuum and autoradiography

was performed using preflashed Curix RP-2 films exposed for 72 hours between intensifying screens at -70°C .
Protein determination: The method of Bradford (16) was employed using bovine serum albumin as a standard . Protein content of columns was monitored at 280 nm.

Results

Inhibition of NADPH oxidase activation by dialdehyde nucleotides. NADPH oxidase was activated by incubation of neutrophil membranes, cytosol and arachidonate (14) . In agreement with earlier reports (3-6) when activation was conducted in the presence of GDP , a dose dependent inhibition of the enzymic activity was observed (Fig.1) . At similar concentrations , GTP also interfered with activation, most probably due to the presence of GDP formed by its hydrolysis ; $NADP^+$ was essentially inactive (Fig.1). Qualitatively similar though more pronounced effects were observed when the nucleotides were replaced by their dialdehyde analogs (oGDP, oGTP, or $oNADP^+$) (Fig.1) . Since $oNADP^+$ had no effect on activation of the oxidase , it was concluded that the guanosine portion of the dialdehyde derivatives retained its specificity towards oxidase-activating system .

Activation of the oxidase in the absence and presence of GDP or oGDP was carried out at increasing concentrations of GTPγS (Fig.2). The presence of GDP and oGDP shifted the GTPγS dose response curves to the right, consistent with competition of the natural and dialdehyde GDP with GTPγS for the site on the oxidase activating protein (Fig.2).
Irreversible inhibition of NADPH oxidase activating cytosolic activity by oGDP. On the basis of experiments shown in Figs.1 and 2 , oGDP was chosen as an affinity analog of GDP to be tested separately with cytosolic or membrane neutrophil fractions for irreversible inhibition . After incubation with oGDP (1 hour), cytosol was chromatographed on a Sephadex G-25 column to remove unbound nucleotides and tested for its

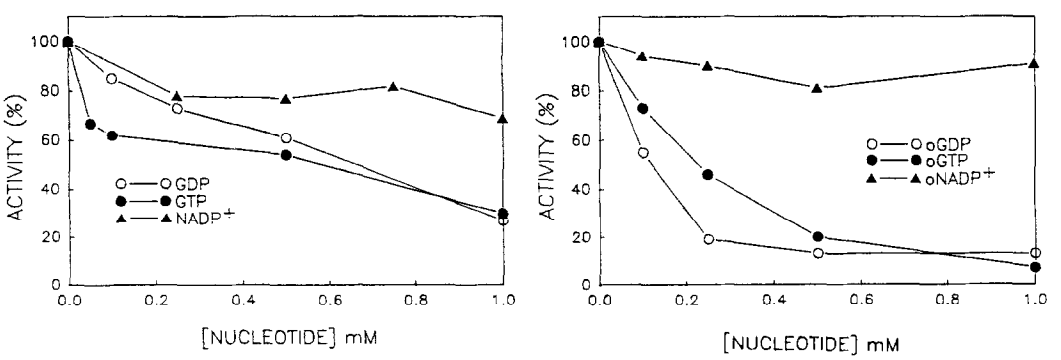

Fig.1. The effect of nucleotides and their dialdehyde analogs on the NADPH oxidase activity. Nucleotides were added to the activation mixture. Control (100%) activity was 420 nmol/min.mg membrane protein.

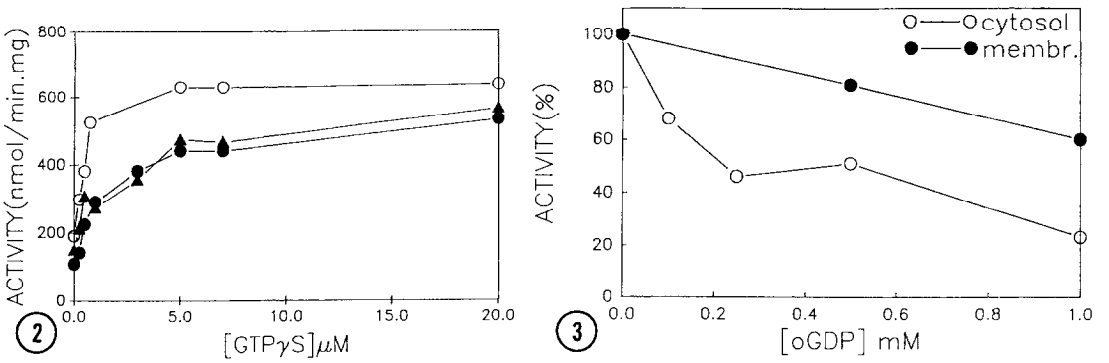

Fig.2. Dose response of the enhancement of NADPH oxidase activity by GTPγS : activation was carried out (o) without additions ; (▲)- in the presence of 0.1 mM GDP and (●) 0.1 mM oGDP.

Fig.3. Irreversible inhibition of the capacity of cytosol and membranes to support NADPH oxidase activation by oGDP. Cytosol or membranes were incubated (room temperature,1 hour) with oGDP and freed of unbound nucleotide by gel filtration . Residual activity was determined in the presence of 7μM GTPγS.

capacity to support cell-free activation in the presence of GTPγS. Treatment with oGDP in a dose-dependent manner reduced the capacity of cytosol to support activation of the oxidase (Fig. 3). When 1 mM borohydride was included during the incubation with oGDP, a similar extent of inhibition was observed, implying that reduction of a Schiff base was not essential for the irreversible blockade of oxidase-linked component by dialdehyde (data not shown) (10,11). Membranes incubated with oGDP lost a fraction of their oxidase- supporting activity (Fig.3); this effect was weaker and was not further investigated .

Specificity of the irreversible inhibition of cytosol by oGDP was tested by substitution of the latter by oGTP and oNADP$^+$. Both analogs of guanosine were potent inhibitors of the oxidase-activating system blocking 55.4±6.4% and 41.0±1.0% of cytosolic activity respectively; the inhibition by oNADP was negligible (8.5±0.5%).

The effect of oGDP on fractionated cytosol . Untreated cytosol was fractionated by successive chromatography on S and Q Sepharose columns (Fig.4). Flowthrough of the first i.e. S Sepharose column (S150), was transferred to a Q Sepharose column while bound proteins were eluted with a linear NaCl gradient. Chromatography of S150 on Q Sepharose resulted in its separation into two active fractions : the Q Sepharose flowthrough (Q150) and a fraction eluting at 0.3M NaCl (Q300) . As shown in Fig.4, combination of Q150 , Q300 and S350 with arachidonate and GTPγS synergistically supported reconstitution of oxidase activity. Immunoblotting with antibodies produced against oxidase linked cytosolic components, p47 and p67 revealed the presence of p47 in fraction S350 and of p67 in Q300 (data not shown) (8).

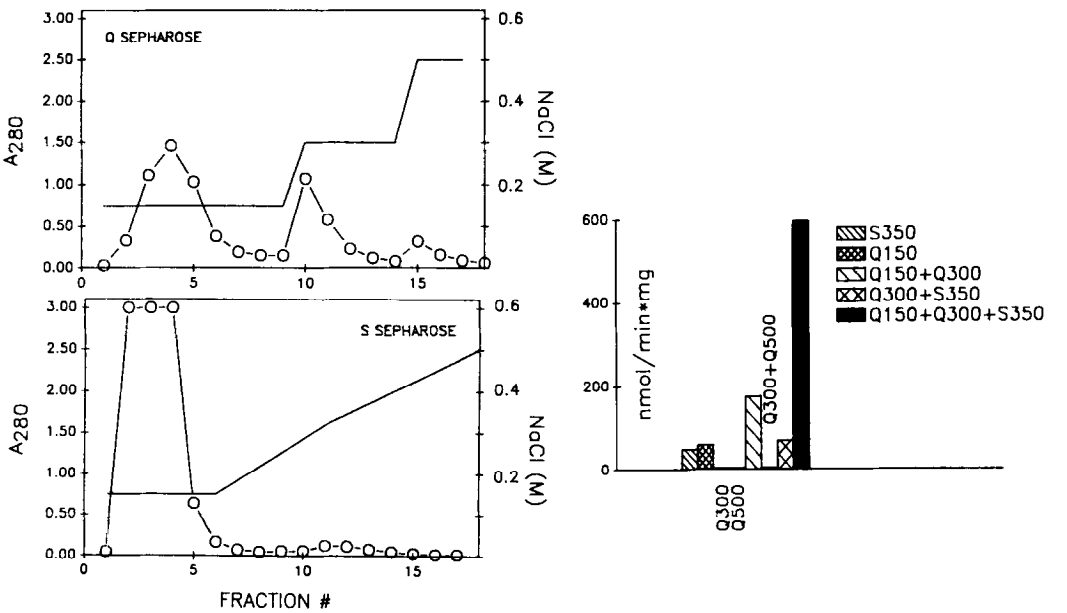

Fig.4. Fractionation of neutrophil cytosol on ion exchanger columns and reconstitution of oxidase activity by combination of fractions . Cytosol (2 ml) was loaded on S Sepharose. The flowthrough was transferred and fractionated on a Q Sepharose column . The right part depicts oxidase activity of different combinations of the fractions as indicated.

To identify the fraction interacting with oGDP, all three fractions (S350, Q150 and Q300) were incubated separately in the presence and absence of 0.1mM oGDP. The capacity of each fraction to reconstitute NADPH oxidase activity in combination with the two other untreated fractions was then determined. It was found that oGDP-treated S350 and Q300 fractions lost ca.50% of their activity while Q150 was not affected (Table I) . These results implicated two out of three cytosolic NADPH oxidase-activating fractions in the interaction with the dialdehyde.

Labeling of oGDP/oGTP binding components. Dialdehyde-GDP in our experiments blocked irreversibly oxidase-supporting activity without the need for borohydride to reduce the Schiff base (10,11), suggesting involvement of a dihydroxymorpholino type of adduct (12,13) . In view of this, attempts to label the component interacting with oGDP were made employing oxidized in situ [α^{32}P]-GTP in the absence of borohydride. Unfractionated cytosol treated with oGTP revealed several distinct oGTP adducts on the autoradiograms (Fig.5). In autoradiogram of fraction S350 , bands migrating as 24 and 46 kDa proteins were labeled. In the Q300 column fraction a number of weakly radiolabeled bands including a 64 kDa protein were detected (Fig.5).

Discussion

The irreversible blockade of the capacity of unfractionated neutrophil cytosol to support cell-free activation of NADPH oxidase by

Table I
Inhibition of oxidase fractions by oGDP[a]

Fraction	nmol O_2 /min.mg	
	no oGDP[b]	plus oGDP[c]
S350	290	154
Q150	283	259
Q300	273	157

[a]Reconstituted activity of the three combined fractions kept on ice equaled 358 nmol/min.mg.[b]the indicated fraction was incubated for 1h at room temperature and combined with two other fractions kept on ice.[c]the indicated fraction was incubated with 100μM oGDP for 1h at room temperature and combined with two other fractions kept on ice.

dialdehyde-GDP (Fig.3) is consistent with participation of a cytosolic component/s in modulation of oxidase activity by guanine nucleotides (3-6). The finding that oNADP$^+$ had only a minor inhibitory effect implies that the inhibition reported here should not be ascribed to a nonspecific reaction of the dialdehyde group of the analogs.

It is noteworthy that the two oxidase-linked fractions, S350 and Q300 inactivated by the dialdehyde analog, contain the two recently characterized oxidase subunits, p47 and p67 respectively (7-9). Inhibition of both fractions by oGDP may be attributed to the presence of oGDP-binding sites on p47 and p67 in agreement with the binding of both subunits to GTP-agarose (7). Moreover, the inhibition suggests that these sites may be essential for activity. It should be pointed out that while p47 and p67 bear no resemblance to classical G proteins and do not contain sequences specific for GTP specific binding sites (8,9), their function in the oxidase complex is not understood.

In labeling experiments carried out with isolated cytosolic fractions (Fig.5), incorporation of [α-^{32}P]-oGTP into 46 kDa and 64 kDa proteins in both oGDP-inhibitable fractions, S350 and Q300 respectively, was indeed observed, consistent with the existence of an oGDP/oGTP-binding site on p47 and p67. Since however in fractions S350

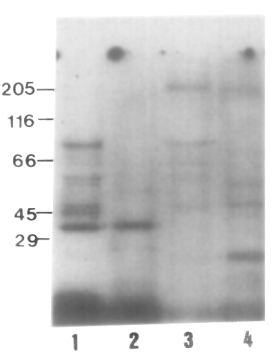

Fig.5. Autoradiogram of cytosol and ion exchanger fractions preincubated in the presence of [α^{32}]-oGTP and run on 5-15% SDS-polyacrylamide gels. Lane 1 - crude cytosol; lane 2 - Q150; lane 3 - Q300; lane 4 - S350.

and Q300, several additional proteins were radiolabeled, inhibition caused by the reaction of oGDP with other components cannot be ruled out. Moreover, it has been recently suggested by Okamura et al.(17) that p47 and p67 of the resting cytosol form as a complex which may incorporate also other oxidase-linked component/s. It is conceivable that a guanine nucleotide binding subunit belongs to the complex and coelutes with p47 and p67 in fractions S350 and/or Q300 respectively. This assumption explains the inhibition of both fractions by oGDP without implicating two GTP-binding sites . Thus while our experiments demonstrate a possible functional correlation between binding of guanine nucleotides and the activity of p47 and p67, inactivation of ion exchanger column fractions S350 and Q300 by the action of oGDP on hitherto unidentified protein/s contained in these fractions cannot at present be ruled out . Furthermore, our experiments do not exclude the possibility that membrane GTP-binding proteins may also be involved in the regulation of NADPH oxidase activity (18).

Acknowledgements

We thank Dr. H.L. Malech for probing our blots with his anti-p47 and anti-p67 antibodies. This research was supported in part by The Binational U.S.-Israel Science Foundation.

References

1. Curnutte, J.T., and Babior, B.M.(1987) Adv. Hum. Genet. 16,229-297.
2. Segal, A.W.,(1989) J.Clin.Invest.83, 1785-1793.
3. Seifert,R., Rosenthal,W., and Schultz, G. (1986) FEBS Letters 205, 161-165.
4. Gabig, T.G., English, D., Akard, L.P. and Schell, M.J. (1987) J.Biol.Chem. 262,1685-1690.
5. Ligeti, E., Doussiere, J., and Vignais, P.V. (1988) Biochemistry 27, 193-200.
6. Ligeti, E., Tardif,M., and Vignais, P.V. (1989) Biochemistry 28, 7116-7123.
7. Volpp, B.D., Nauseef, W.M., and Clark, R.A. (1988) Science 242,1295-1297. 8
8. Lomax, K.J., Leto,T.L., Nunoi,H., Gallin, J.I., and Malech, H.L. (1989) Science 245, 409-412.2
9. Leto, T.L., Lomax, K.J., Volpp, B.D., Nunoi, H., Sehlar,J.M.G., Nauseef, W.M., Clark, R.A.,Gallin, J.I., Malech, H.L.(1990) Science 248,727-730.
10. Estabrook-Smith, S.B., Wallace,J.C., and Keech, D.B., (1976) Eur. J. Biochem.62, 125-130.
11. Colman, R.F.(1983) Ann. Rev. Biochem.52, 67-91.
12. King, M.M. and Carlson, G.M. (1981) Biochemistry 20, 4382-4387.
13. White, B.J. and Levy,H.R. (1987) J. Biol. Chem. 262, 1223-1229.
14. Aviram. I. and Sharabani M. (1989) Biochem. J. 261, 477-482.
15. Pilloud, M.-C., Doussiere,J., and Vignais,P.V. (1989) FEBS Letters 257,167-170.
16. Bradford, M. (1976) Anal. Biochem. 72, 248-254.
17. Okamura, N., Babior, B.M., Mayo, L.A.,Smith, R.M., and Curnutte,J.T. (1990) J. Clin. Invest. 85, 1583-1587.
18. Quinn,M.T., Parkos, C.A., Walker, L., Orkin,S.H., Dinauer, M.C., and Jesaitis,A.J. (1989) Nature 342, 198-200.