Involvement of GTP in cell-free activation of neutrophil NADPH oxidase

Studies with GTP analogues

Ety KLINGER and Irit AVIRAM*

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

Activation of superoxide-producing NADPH oxidase of neutrophils requires the presence of cell membranes, cytosolic components and arachidonate and is markedly enhanced by non-hydrolysable analogues of guanine nucleotides, i.e. guanosine 5'-[γ -thio]triphosphate and guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG). Gel filtration and ultrafiltration of the cytosol decreased the basal activity of NADPH oxidase. Activity could be restored by GTP, suggesting participation of the nucleotide in basal activation. Preincubation of neutrophil cytosol with periodate-oxidized p[NH]ppG (ox-p[NH]ppG) followed by gel filtration resulted in a time-dependent enhancement of basal oxidase activity. The presence of GDP or GTP, but not ATP, during the incubation with ox-p[NH]ppG abolished this enhancement. These data are consistent with a stable association of ox-p[NH]ppG with an oxidase-linked cytosolic protein. SDS/PAGE of neutrophil cytosol preincubated with [3 H]ox-p[NH]ppG revealed radioactivity in bands migrating as 100, 70, 47, 34 and 22 kDa proteins. Evidence for covalent labelling of the cytosolic protein p47-phox with [3 H]ox-p[NH]ppG is presented. Heterogeneity of cytosolic GTP-binding sites and possible participation of protein p47-phox in functional interaction with GTP analogues during cell-free activation are suggested.

INTRODUCTION

The superoxide-generating NADPH oxidase of neutrophils [1,2] can be activated *in vitro* by exposure of plasma membranes to unsaturated fatty acids or anionic detergents [3,4]. In addition to membranes containing oxidase-associated cytochrome *b*-558, activation of the enzyme requires the presence of neutrophil cytosol. The latter supplies at least three protein components essential for activation or activity of the oxidase [5–8]. Two oxidase-linked cytosolic proteins, p47-phox and p67-phox, have recently been characterized and cloned, but their function has not yet been defined [9,10].

The basal activity of NADPH oxidase activated in a mixture of membranes, cytosol and arachidonate can be augmented by the addition of poorly hydrolysable analogues of GTP, guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG) or guanosine 5'-[γ -thio]triphosphate (GTP[S]) [11,12], implicating membrane and/or cytosolic GTP-binding proteins in the process. The question of the location and identity of GTP-modulated component(s) of the oxidase has been addressed by several investigators [7,13–15]. Volpp *et al.* [5] identified oxidase subunits bound to GTP-agarose as cytosolic proteins p67-phox and p47-phox, but the functional relevance of this interaction was not clarified. Bolscher *et al.* [7] presented evidence for the dependence of activity of the third cytosolic oxidase-linked component, SOC 1, on GTP[S]. Co-purification of a membrane-bound low-molecular-mass GTP-binding protein, rap 1, with cytochrome *b*-558 of the oxidase led to speculations regarding its participation in the NADPH oxidase complex activity [16].

In a recent study we employed periodate-oxidized dialdehyde derivatives of GDP and GTP as affinity labels for the GTP-binding site involved in modulation of oxidase activity [17]. Our finding that dialdehyde-GDP blocked the capacity of cytosol to activate NADPH oxidase implicated cytosolic guanine-nucleotide-binding site(s) in modulation of the enzyme.

In the present paper we show that the basal arachidonate-mediated activity of NADPH oxidase requires exogenous GTP. We describe formation of a stable high-affinity complex of a component of neutrophil cytosol with a periodate-oxidized analogue of p[NH]ppG, ox-p[NH]ppG. This interaction leads to a time-dependent augmentation of basal oxidase activity and is reflected in labelling of several cytosolic proteins, including a 48 kDa protein immunoprecipitated by antiserum to p47-phox. Preliminary results of this study have been reported [18].

After submission of the present paper, two reports on the participation of low-molecular-mass G-proteins in the activation of NADPH oxidase were published [19,20]. Eklund *et al.* [19] implicated Krev-1 or a related low-molecular-mass G-protein in the process. Abo *et al.* [20] identified the two subunits of σ 1, the third cytosolic component of guinea-pig macrophage NADPH oxidase [8], as p21rac1 and its modulator, GDP-dissociation inhibitor, rho GDI. Notwithstanding these findings, the mode of activation of NADPH oxidase by guanine nucleotides has not yet been delineated.

MATERIALS AND METHODS

Materials

Chemicals, nucleotides, immunochemicals and resins were purchased from Sigma.

Fractionation of neutrophils

Human neutrophils were isolated from fresh buffy coats by standard procedures. Cells (10^8 /ml) in 10 mM-potassium phosphate-buffered saline (pH 7.0) (PBS) containing 1 mM-EGTA and proteinase inhibitors were disrupted by sonication and fractionated into soluble cytosolic and light membrane fractions as described [17,21].

Abbreviations used: p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; ox-p[NH]ppG, periodate-oxidized p[NH]ppG; GTP[S], guanosine 5'-[γ -thio]triphosphate; PBS, phosphate-buffered saline (0.131 M-NaCl/10 mM-potassium phosphate, pH 7.0).

* To whom correspondence should be addressed.

NADPH oxidase activation and assay

Arachidonate-dependent activation was performed in two successive steps [21]. The optimal arachidonate concentration was determined for each experiment.

Gel filtration and ultrafiltration of the cytosol

Cytosol (0.25 ml) was chromatographed on a Sephadex G-25 column (0.5 cm $\times$ 20 cm) equilibrated with 10 mM-Hepes (pH 6.8)/0.13 M-NaCl/0.5 mM-MgCl₂. Fractions of volume 1 ml were collected and their A_{280} was determined. The volume of the protein-containing fraction used for oxidase activation was corrected for the dilution on the column. Ultrafiltration was carried out in Centricon-10 (Amicon) microconcentrators.

Treatment of the cytosol with ox-p[NH]ppG

ox-p[NH]ppG was prepared as described [22] and stored at -70°C . Occasionally, it exhibited instability during storage. The concentration of ox-p[NH]ppG was determined at 259 nm by using $\epsilon_{\text{mm}} = 15.9$. Cytosol was incubated for the desired period with 10 μM -ox-p[NH]ppG and separated from unbound reagent on a Sephadex G-25 column.

Labelling with [³H]ox-p[NH]ppG

[³H]p[NH]ppG (25 μM ; 19 Ci/mmol; Amersham) was oxidized with 30 μM -NaIO₄ (1 h at 0°C in the dark) followed by 15 μM -ethylene glycol [22]. Cytosol was mixed with [³H]ox-p[NH]ppG (5 μM) and incubated for 2 h at 26°C . Proteins were precipitated with 10% (w/v) trichloroacetic acid and the precipitates were washed with 1 ml of cold acetone (three times), dissolved in SDS/PAGE sample buffer and subjected to electrophoresis in 7–15% gradient gels [23]. Prestained molecular-mass markers (Bio-Rad) were run in parallel. Gels were cut into 2.5 mm slices, treated overnight with 30% (v/v) H₂O₂ at 42°C and counted for radioactivity in a liquid-scintillation counter.

Fractionation of cytosol on an S Sepharose column

The concentration of NaCl in the cytosol was decreased to 50 mM by ultrafiltration in Centricon 10 membranes (Amicon) and subsequent dilution with 10 mM-potassium phosphate, pH 7.0. Cytosol (2×10^8 cell equivalents) was loaded (12 ml/h) on a 1.0 ml S Sepharose column equilibrated with 50 mM-NaCl/20 mM-Hepes, pH 7.5. After the column had been washed, a 10 ml gradient (0.05–0.5 M-NaCl) was applied and 1 ml fractions were collected. Protein content was estimated from the A_{280} . A portion (50 μl) of each fraction was subjected to SDS/PAGE and immunoblot analysis.

Immunoblot analysis

Proteins on SDS/polyacrylamide gels were transferred to nitrocellulose in 192 mM-glycine/25 mM-Tris (pH 8.3)/20% (v/v) methanol by using a semi-dry transfer unit (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). Nitrocellulose was blocked with 2% BSA/3% non-fat dry milk in PBS (90 min) and incubated overnight in the cold with a 1:1 mixture of goat antisera to recombinant proteins p47-phox and p67-phox (1:1000) [9,10], a gift from Dr. H. L. Malech and Dr. T. Leto; the blots were washed (three times) with PBS and PBS/0.05% Triton X-100 and developed with peroxidase-conjugated rabbit anti-goat IgG, with 4-chloronaphthol and 0.4 mM-H₂O₂ as substrates.

Immunoprecipitation of [³H]ox-p[NH]ppG-labelled cytosolic proteins fractionated on S Sepharose

S Sepharose fractions showing the presence of both p47-phox and p67-phox proteins (fractions 10–12) were pooled and concentrated in Centricon-10 units. The NaCl concentration was

adjusted to 0.15 M. Proteins in the pooled fraction were labelled with 5 μM -[³H]ox-p[NH]ppG, diluted with an equal volume of 150 mM-NaCl/10 mM-EDTA/1% deoxycholate/1% Nonidet P40/10 mM-Tris/HCl, pH 7.4, and incubated (2 h on ice) with 2 μl of goat antisera to proteins p47-phox and p67-phox [24]. Protein A-agarose beads (0.25 mg) pretreated with neutrophil cytosol (10^8 cell equivalents; 2 h at 4°C) were added and the mixture was incubated overnight at 4°C , centrifuged and washed as described by Rotrosen *et al.* [24]. The washed beads were resuspended in SDS/PAGE sample buffer, boiled for 3 min and pelleted. Supernatants were analysed by SDS/PAGE in minigels (7–15% gradient gels). Radioactivities of sliced gels were determined as described above. In control experiments radiolabelled S Sepharose fractions were incubated in the absence of goat serum or with normal goat serum.

Protein determination

Protein was estimated by the method of Bradford [25] with BSA as standard.

RESULTS

Membranes activated in the presence of arachidonate and unfractionated cytosol exhibit basal superoxide production rates which undergo a 2–5-fold enhancement by the inclusion of GTP[S] or p[NH]ppG in the activation mixture [11,12,19,21]. In this respect, ox-p[NH]ppG resembled the native nucleotide and augmented oxidase activity, albeit to a lesser extent (Fig. 1).

When cytosol used for activation of NADPH oxidase was depleted of low-molecular-mass substances by gel filtration on a Sephadex G-25 column (Table 1), a considerable decrease in basal oxidase activity was observed. Gel filtration had only a minor effect on the activity estimated after activation in the presence of GTP[S]. Ultrafiltration of the cytosol through a 10 kDa-cut-off filter or dialysis also led to a diminution of basal activity (results not shown). These data imply that a low-molecular-mass substance(s) was essential for basal oxidase activation. Indeed, the decreased basal activity could be partially restored by reconstitution with the low-molecular-mass fraction from the gel-filtration column (Table 1) or with the ultrafiltration filtrate (results not shown). The activity-restoring capacity of these fractions was not affected by boiling. Low concentrations of GTP also restored basal activity (Table 1). The effect of GTP was dose-dependent, with saturation above 2 μM (results not shown). These results suggest that cytosolic GTP participates in basal NADPH oxidase activation.

The basal activity of cytosol preincubated with p[NH]ppG (Fig. 2) or GTP[S] (results not shown) was similarly decreased by

Fig. 1. Enhancement of NADPH oxidase activation by the addition of p[NH]ppG (○) and ox-p[NH]ppG (●) to the activation mixture

Results shown are means $\pm$ S.D. of five experiments.

Table 1. Effect of gel filtration of cytosol on NADPH oxidase activity

Cytosol

NADPH oxidase activity (nmol/min per mg of protein)

Before gel filtration

Basal activity 602 ± 84 (n = 10)

+ GTP[S] (7 μM) 1561 ± 301 (n = 10)

After gel filtration

Basal activity 164 ± 91 (n = 10)

+ 7 μM-GTP[S] 1190 ± 377 (n = 5)

+ low-molecular-mass fraction 438 ± 150 (n = 4)

+ boiled (10 min) low-molecular-mass fraction 397 (n = 2)

+ 10 μM-GTP 385 ± 81 (n = 4)

Fig. 2. Effect of preincubation of neutrophil cytosol with 10 μM-ox-p[NH]ppG (●) or ox-p[NH]ppG (○) on cell-free NADPH oxidase activation

Preincubation was terminated by gel filtration of the cytosol as described in the text. The results are means ± S.D. of three experiments.

gel filtration. However, when cytosol was preincubated for 0–5 h with 10 μM-ox-p[NH]ppG and chromatographed on Sephadex G-25, a time-dependent increase in the basal activity was observed (Fig. 2). These data are consistent with a stable association of ox-p[NH]ppG with a cytosolic component of NADPH oxidase. Superoxide production by NADPH oxidase activated in the presence of p[NH]ppG was not significantly affected by gel filtration or by preincubation of cytosol with p[NH]ppG or its dialdehyde analogue (Table 2).

The specificity of interaction of the putative cytosolic protein with ox-p[NH]ppG was established by inclusion of a 100-fold excess of GTP, GDP or ATP in the incubation mixture containing ox-p[NH]ppG. As shown in Table 2, GTP and GDP prevented enhancement of basal activity by ox-p[NH]ppG, consistent with the competition with the latter for the same binding site; ATP was ineffective. GDP or GTP added after preincubation with ox-p[NH]ppG, i.e. before loading on to the gel-filtration column, were unable to interfere with the enhancement of the activity (results not shown).

In cytosol treated with [³H]ox-p[NH]ppG (5 μM) and subjected to SDS/PAGE, several radioactive bands migrating as 100, 70, 47, 34 and 22 kDa proteins were detected (Fig. 3). Experiments

Table 2. Competition of GTP, GDP and ATP with ox-p[NH]ppG

ox-p[NH]ppG (10 μM) was preincubated with cytosol for 1 h in the absence or presence of 1 mM-GTP, -GDP or -ATP. Activation of NADPH oxidase was carried out in a 0.2 ml volume in the absence (basal activity) or presence of 10 μM-p[NH]ppG. The data are mean values of representative experiments performed in duplicate. The experiments were repeated at least three times.

NADH oxidase activity (nmol/min per mg of protein)

Treatment of cytosol

Basal + p[NH]ppG

Experiment 1

None 231.0 976.5

Gel filtration 87.5 1015.0

ox-p[NH]ppG (1 h), gel filtration 308.0 920.5

ox-p[NH]ppG + GDP (1 h), gel filtration 80.5 980.0

ox-p[NH]ppG + GTP (1 h), gel filtration 108.5 1127.0

Experiment 2

None 563.0 1400.0

Gel filtration 105.0 1330.0

ox-p[NH]ppG (1 h), gel filtration 210.0 1290.0

ox-p[NH]ppG + ATP (1 h), gel filtration 253.0 1230.0

Fig. 3. SDS/PAGE of cytosolic proteins labelled with 5 μM-[³H]ox-p[NH]ppG

The positions of prestained molecular-mass markers (kDa) are indicated.

were devised to find out whether the 47 and 70 kDa labelled bands were related to proteins p47-phox and p67-phox of the oxidase. For this purpose, the two oxidase-linked proteins were partially purified by chromatography of the cytosol on S Sepharose cation exchanger and identified in the column fractions by immunoblot analysis with specific antibodies (Fig. 4). The pooled fractions were concentrated by ultrafiltration, labelled with 5 μM-[³H]ox-p[NH]ppG and proteins p47-phox and p67-phox were immunoprecipitated. The precipitates were resolved by SDS/PAGE, and the gels were sliced and their radioactivities determined as shown in Fig. 5. The main radioactive peak of the immunoprecipitate migrated as a 48 kDa protein. A labelled peak of similar mobility was observed after immunoprecipitation of [³H]ox-p[NH]ppG-labelled cytosol; the peak was absent from controls in which normal goat serum was substituted for anti-serum to proteins p47-phox and p67-phox (results now shown),

Fig. 4. Fractionation of neutrophil cytosolic proteins on S Sepharose

○, A_{280} ; ----, NaCl concentration. Inset: immunoblot analysis of proteins in column fractions resolved by SDS/PAGE and developed with antibodies to proteins p47-phox and p67-phox. This is a representative experiment repeated six times with similar results.

Fig. 5. SDS/PAGE of pooled cytosolic fractions (9-11) from an S Sepharose column labelled with $5 \mu\text{M}$ - $[\text{3H}]$ ox-p[NH]ppG and immunoprecipitated with a mixture of antibodies to proteins p47-phox and p67-phox

The electrophoretic migration of proteins p47-phox and p67-phox of untreated cytosol transferred to nitrocellulose and developed with specific antibodies is shown in the inset.

implying identity with p47-phox. The small difference in the position of the radiolabelled peak in comparison with that of protein p47-phox of untreated cytosol identified by antibodies after transfer to nitrocellulose (Fig. 5) may be attributed to the technical difficulty in alignment of the immunoblot with the sliced gel. The labelling of protein p47-phox implies a high-affinity association of the radiolabelled guanine nucleotide with the protein.

DISCUSSION

The gel-filtration and ultrafiltration experiments (Table 1) imply that basal activation of NADPH oxidase depends on endogenous GTP. These results are in keeping with the previously reported diminution of cytosolic oxidase-supporting activity in aged cytosol, attributed to the depletion of endogenous cytosolic GTP [12]. However, they contradict recent data of Gabig *et al.* [15] showing that the basal activity of the oxidase was not affected by gel filtration of the cytosol. These discrepancies may be attributed to difference in experimental conditions, e.g.

concentration of Mg^{2+} present during incubations and gel filtration [26], or to different types of assays [15,21].

Loss of basal activity on gel filtration was not prevented by pre-exposure of cytosol to p[NH]ppG (Fig. 2). Apparently, the oxidase-linked GTP-binding cytosolic protein did not bind the nucleotide analogue under these conditions. Alternatively, p[NH]ppG was bound and dissociated after the unbound ligand had been depleted by gel filtration. The time-dependent augmentation of the basal activity of NADPH oxidase observed after pretreatment of cytosol with $10 \mu\text{M}$ -ox-p[NH]ppG (Fig. 2) implies a stable association of ox-p[NH]ppG with a cytosolic component. The specificity of this reaction for guanine nucleotides was demonstrated by competition of ox-p[NH]ppG with GTP and GDP, but not with ATP (Table 2). To our knowledge, this is the first demonstration of functional binding of a guanine analogue to an oxidase-linked component of neutrophil cytosol in the absence of membranes [7] or arachidonate [15].

These results corroborate our previous findings on the modification of cytosolic oxidase component(s) by dialdehyde-guanine nucleotides [17]. In all cases, dialdehyde analogues exerted their activity in the absence of NaBH_4 , which is required for the stabilization of Schiff bases [22], implicating other types of stable linkage, e.g. dihydroxymorpholino compounds [27,28]. Oxidized GDP, however, inhibited activity [17], whereas ox-p[NH]ppG, used in the present study, prevented loss of basal activity. Moreover, the latter was effective at micromolar concentrations, implying high affinity and specificity of binding.

The effect exerted by a long incubation with ox-p[NH]ppG could be further potentiated by a non-hydrolysable guanine nucleotide (Table 2), suggesting that vacant nucleotide-binding sites were still available for interaction with stable guanine nucleotides during activation of the NADPH oxidase. This is compatible with the existence of two fractions of oxidase-modulating GTP-binding sites in the cytosol: one fraction consists of sites interacting with ox-p[NH]ppG in the absence of arachidonate and/or membranes. Binding of ox-p[NH]ppG to these sites is responsible for the partial preservation of oxidase activity after gel filtration (Fig. 2). Binding of p[NH]ppG or GTP[S] to residual sites may require conditions suitable for activation, e.g. the presence of arachidonate and/or membranes [7,15]. It is conceivable that this apparent heterogeneity of the binding sites may reflect binding sites located on distinct proteins. A kinetic heterogeneity of NADPH oxidase activatable in an arachidonate-dependent cell-free system has previously been documented: it was shown that basal activation estimated in the absence of exogenously added GTP followed a first-order reaction course, whereas GTP[S]-enhanced activation was sigmoid and slower [21].

In our experiments several cytosolic proteins were labelled with $[\text{3H}]$ ox-p[NH]ppG, including proteins similar in size to small G-proteins (Fig. 3). The recent studies by Eklund *et al.* [19] and Abo *et al.* [20] implicated Krev-1 and p21rac1 G-proteins in the activation of NADPH oxidase in human neutrophil and guinea-pig macrophage cytosols respectively. The complexity of the involvement of guanine nucleotides in the activity of NADPH oxidase is demonstrated by the findings that, in the experimental system of Eklund *et al.* [19], depletion of cytosolic nucleotides by gel filtration of the cytosol had no effect on its basal oxidase-activating power [15], whereas the activity of purified $\sigma 1$ in the SDS-mediated cell-free oxidase assay used by Abo *et al.* [20] was independent of guanine nucleotides. Moreover, $\sigma 1$ did not bind to GTP-agarose and other nucleotide-bound resins [8] and was not inhibited by oxidized GDP [29]. Thus, in spite of the dependence of oxidase activation on the presence of small G-proteins, a functional interaction of ox-p[NH]ppG (Fig. 2) with these proteins seems unlikely. Immunoprecipitation

of a [^3H]ox-p[NH]ppG-labelled 48 kDa protein by anti-(p47-phox) (Fig. 5) implicated p47-phox in covalent modification by ox-p[NH]ppG and suggested p47-phox as a possible target of modulation by ox-p[NH]ppG. This hypothesis is consistent with the previously reported binding of protein p47-phox to resin-bound nucleotides [5,30] and with blockade of the oxidase activation-supporting activity of column fractions containing protein p47-phox by the dialdehyde analogue of GDP, oxidized GDP [17]. It is noteworthy that the exact role of protein p47-phox in the activity/activation of the NADPH oxidase complex has not been elucidated [31–34]. Since it does not possess structural features characteristic of a GTP-binding site [9], corroboration or refutation of the suggested participation of the protein in binding of guanine nucleotides must await reconstitution of basal oxidase activity from purified components.

This work was supported in part by the Israel–U.S.A. Binational Science Foundation grant. We thank Dr. H. L. Malech and Dr. T. L. Leto for antisera to p47-phox and p67-phox.

REFERENCES

- Curnutte, J. T. & Babior, B. M. (1987) *Adv. Hum. Genet.* **16**, 229–297
- Segal, A. W. (1989) *J. Clin. Invest.* **83**, 1785–1793
- Heyneman, R. A. & Vercauteren, R. E. (1984) *J. Leukocyte Biol.* **36**, 751–759
- Bromberg, Y. & Pick, E. (1985) *J. Biol. Chem.* **260**, 13538–13545
- Volpp, B. D., Nauseef, W. M. & Clark, R. A. (1988) *Science* **242**, 1295–1297
- Nunoi, H., Rotrosen, D., Gallin, J. I. & Malech, H. L. (1988) *Science* **242**, 1298–1301
- Bolscher, B. G. J. M., Denis, S. W., Verhoeven, A. J. & Roos, D. (1990) *J. Biol. Chem.* **265**, 15782–15787
- Abo, A. & Pick, E. (1990) *Eur. J. Clin. Invest.* **20**, A37
- Lomax, K. J., Leto, T. L., Nunoi, H., Gallin, J. I. & Malech, H. L. (1989) *Science* **245**, 409–412
- 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
- Gabig, T. G., English, D., Akard, L. P. & Schell, M. J. (1987) *J. Biol. Chem.* **262**, 1685–1690
- Ligeti, E., Doussiere, J. & Vignais, P. V. (1988) *Biochemistry* **27**, 193–200
- Doussiere, J., Pilloud, M. C. & Vignais, P. V. (1988) *Biochem. Biophys. Res. Commun.* **152**, 993–1001
- Ishida, K., Takeshige, K., Takasugi, S. & Minakami, S. (1989) *FEBS Lett.* **243**, 169–172
- Gabig, T. G., Eklund, E. A., Potter, G. B. & Dykes, J. R., II (1990) *J. Immunol.* **145**, 945–951
- Quinn, M. T., Parkos, C. A., Walker, L., Orkin, S. H., Dinanuer, M. C. & Jesaitis, A. J. (1989) *Nature (London)* **242**, 198–200
- Klinger, E. & Aviram, I. (1991) *Biochem. Biophys. Res. Commun.* **177**, 504–510
- Klinger, E. & Aviram, I. (1991) *Eur. J. Clin. Invest.* **21**, 41
- Eklund, E. A., Marshall, M., Gibbs, J. B., Crean, C. D. & Gabig, T. G. (1991) *J. Biol. Chem.* **266**, 13964–13970
- Abo, A., Pick, E., Hall, A., Totty, N., Teahan, C. G. & Segal, A. W. (1991) *Nature (London)* **353**, 668–670
- Aviram, I. & Sharabani, M. (1989) *Biochem. J.* **261**, 477–482
- Estrabrook-Smith, S. B., Wallace, J. C. & Keech, D. B. (1976) *Eur. J. Biochem.* **62**, 125–130
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Rotrosen, D. E., Kleinberg, M. E., Nunoi, H., Leto, T., Gallin, J. I. & Malech, H. L. (1990) *J. Biol. Chem.* **265**, 8745–8750
- Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
- Hall, A. & Self, A. J. (1986) *J. Biol. Chem.* **261**, 10963–10965
- King, M. M. & Carlson, G. M. (1981) *Biochemistry* **20**, 4382–4387
- White, B. J. & Levy, H. R. (1987) *J. Biol. Chem.* **262**, 1223–1229
- Aharoni, I. (1989) M.Sc. Thesis, Tel-Aviv University
- Leto, T. L., Garrett, M. C., Fujii, H. & Nunoi, H. (1991) *J. Cell Biol.* **111**, 49a
- Clark, R. A., Volpp, B. D., Leidal, K. G. & Nauseef, W. M. (1990) *J. Clin. Invest.* **85**, 714–721
- Tauber, A. I. (1987) *Blood* **69**, 711–720
- Curnutte, J. T., Kuver, R. & Scott, P. J. (1987) *J. Biol. Chem.* **262**, 5563–5569
- Caldwell, S. E., McCall, C. E., Hendricks, C. L., Bass, D. A. & McPhail, L. C. (1988) *J. Clin. Invest.* **81**, 1485–1496

Received 8 August 1991/30 December 1991; accepted 17 January 1992