

## Fatty-acid-induced activation of NADPH oxidase in plasma membranes of human neutrophils depends on neutrophil cytosol and is potentiated by stable guanine nucleotides

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Both *cis* and *trans* unsaturated fatty acids and sodium dodecyl sulfate activated NADPH oxidase in plasma membranes of human neutrophils in the presence of neutrophil cytosol. In contrast, 5,8,11,14-icosatetraenoic acid, saturated fatty acids, esters, peroxides and 4 $\beta$ -phorbol 12-myristate 13-acetate, a potent activator of protein kinase C, were inactive. 5,8,11,14-icosatetraenoic acid inhibited superoxide formation elicited by fatty acids. Guanosine 5'-[ $\gamma$ -thio]triphosphate (GTP[ $\gamma$ S]), a potent activator of guanine-nucleotide-binding proteins (N-proteins) enhanced superoxide formation elicited by fatty acids up to fourfold, supporting our previous suggestion that NADPH oxidase is regulated by an N-protein [Seifert, R. et al. (1986) *FEBS Lett.* 205, 161–165]. Cytosols from various tissues, soybean lipoxygenase and protein kinase C, purified from chicken stomach, did not substitute neutrophil cytosol. The activity of neutrophil cytosol was destroyed by heating at 95°C. Superoxide formation was not affected by the inhibitor of protein kinase C 1-(5-isoquinolinesulfonyl)-2-methylpiperazine (H-7). Removal of cytosolic ATP by preincubation with hexokinase and glucose, dialysis of neutrophil cytosol or chelation of calcium with EGTA did not abolish the stimulatory effect of arachidonic acid and GTP[ $\gamma$ S]. Thus, the cytosolic cofactor appears to be a neutrophil-specific and heat-labile protein, which is neither a lipoxygenase nor protein kinase C.

Binding of the chemotactic peptide, fMet-Leu-Phe, to plasma membrane receptors of neutrophils causes phospholipase-C-mediated degradation of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol 1,4,5-trisphosphate, mobilization of intracellular calcium, release

of arachidonic acid [1–3] and NADPH-oxidase-catalyzed superoxide formation, which has been suggested to be mediated by activation of protein kinase C [4–6]. Recent results indicate that activation of protein kinase C is not crucial for superoxide formation, as the inhibitor of protein kinase C, H-7, did not prevent superoxide generation induced by fMet-Leu-Phe [7, 8]. In neutrophils, depleted of calcium and primed with 4 $\beta$ -phorbol 12-myristate 13-acetate, fMet-Leu-Phe activated NADPH oxidase without stimulation of phosphoinositide hydrolysis and calcium mobilization [9]. As arachidonic acid induces superoxide formation in intact human neutrophils [10, 11] and in cell-free systems [12–14], it was suggested that arachidonic acid or one of its lipoxygenase products may serve as second messenger for activation of NADPH oxidase [14, 15].

Superoxide generation induced by fMet-Leu-Phe, phosphoinositide breakdown and release of arachidonic acid are blocked by treatment with pertussis toxin [1, 2]. We recently reported that arachidonic-acid-induced superoxide formation in plasma membranes of human neutrophils was stimulated up to fourfold by fluoride and the stable GTP analogues guanosine 5'-[ $\gamma$ -thio]triphosphate (GTP[ $\gamma$ S]) and guanosine 5'-[ $\alpha,\beta$ -imido]triphosphate [16], all being potent activators of N-proteins [17]. Our results suggest the direct involvement of an N-protein in the regulation of NADPH oxidase by chemotactic peptides.

In our present study we report that, in plasma membranes of human neutrophils, *cis*- and *trans*-unsaturated fatty acids activate superoxide formation which is potentiated by GTP[ $\gamma$ S]. We present evidence that NADPH oxidase activation is not mediated by either protein kinase C or lipoxygenases.

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Abbreviations. ETYA, 5,8,11,14-icosatetraenoic acid; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; GTP[ $\gamma$ S], guanosine 5'-[ $\gamma$ -thio]triphosphate; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; Me<sub>2</sub>SO, dimethyl sulfoxide; SDS, sodium dodecyl sulfate.

Trivial names. Nervonic acid, *cis*-15-tetracosenoic acid; erucic acid, *cis*-13-docosenoic acid; arachidic acid, *n*-icosanoic acid; homo- $\gamma$ -linolenic acid, 8,11,14-*all-cis*-icosatrienoic acid; arachidonic acid, 5,8,11,14-*all-cis*-icosatetraenoic acid; timnodonic acid, 5,8,11,14,17-*all-cis*-icosapentaenoic acid; stearic acid, *n*-octadecanoic acid; oleic acid, *cis*-9-octadecenoic acid; elaidic acid, *trans*-9-octadecenoic acid; linoleic acid, *cis*-9-*cis*-12-octadecadienoic acid; linolelaidic acid, *trans*-9-*trans*-12-octadecadienoic acid;  $\gamma$ -linolenic acid, 6,9,12-*all-cis*-octadecatrienoic acid; palmitic acid, *n*-hexadecanoic acid; palmitoleic acid, *cis*-9-hexadecenoic acid; palmitelaidic acid, *trans*-9-hexadecenoic acid; myristic acid, *n*-tetradecanoic acid; myristoleic acid, *cis*-9-tetradecenoic acid; lauric acid, *n*-dodecanoic acid.

Enzymes. NADPH oxidase (EC 1.6.99.6); protein kinase C (EC 2.7.1.37); lipoxygenase (EC 1.13.11.12); superoxide dismutase (EC 1.15.1.1).

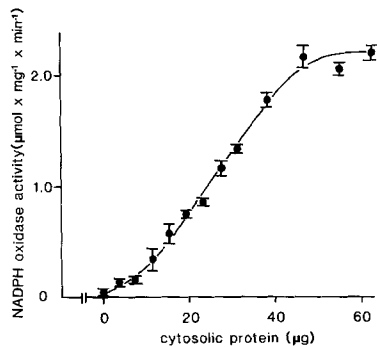


Fig. 1. Dependency of NADPH oxidase activity in neutrophil plasma membranes of neutrophil cytosol. NADPH oxidase in neutrophil plasma membranes (4  $\mu\text{g}$  protein) was stimulated with arachidonic acid (50  $\mu\text{M}$ ) in the presence of different amounts of cytosolic protein (0–65  $\mu\text{g}$  protein). Data represent the mean  $\pm$  SEM of three experiments

## MATERIALS AND METHODS

Human neutrophils from blood of healthy donors were isolated by dextran sedimentation and centrifugation through Ficoll-Hypaque (Biochrom, Berlin, FRG). Cell preparations consisted of more than 98% neutrophils with few monocytes. Cells were disrupted by nitrogen cavitation. The light membrane fraction and cytosol were isolated by centrifugation on discontinuous Percoll gradients (Pharmacia, Uppsala, Sweden) as described previously [6, 18], harvested and stored at  $-80^\circ\text{C}$ . Electron micrographs of light membranes revealed that they consisted of plasma membrane vesicles heterogeneous in size and shape as described recently [18].

NADPH oxidase activity of neutrophil plasma membranes was determined as described [12] by measuring the linear rate of ferricytochrome *c* reduction inhibitable by superoxide dismutase at  $27.5^\circ\text{C}$ . Assays contained 2–12  $\mu\text{g}$  neutrophil plasma membrane protein, 30–160  $\mu\text{g}$  cytosolic protein,

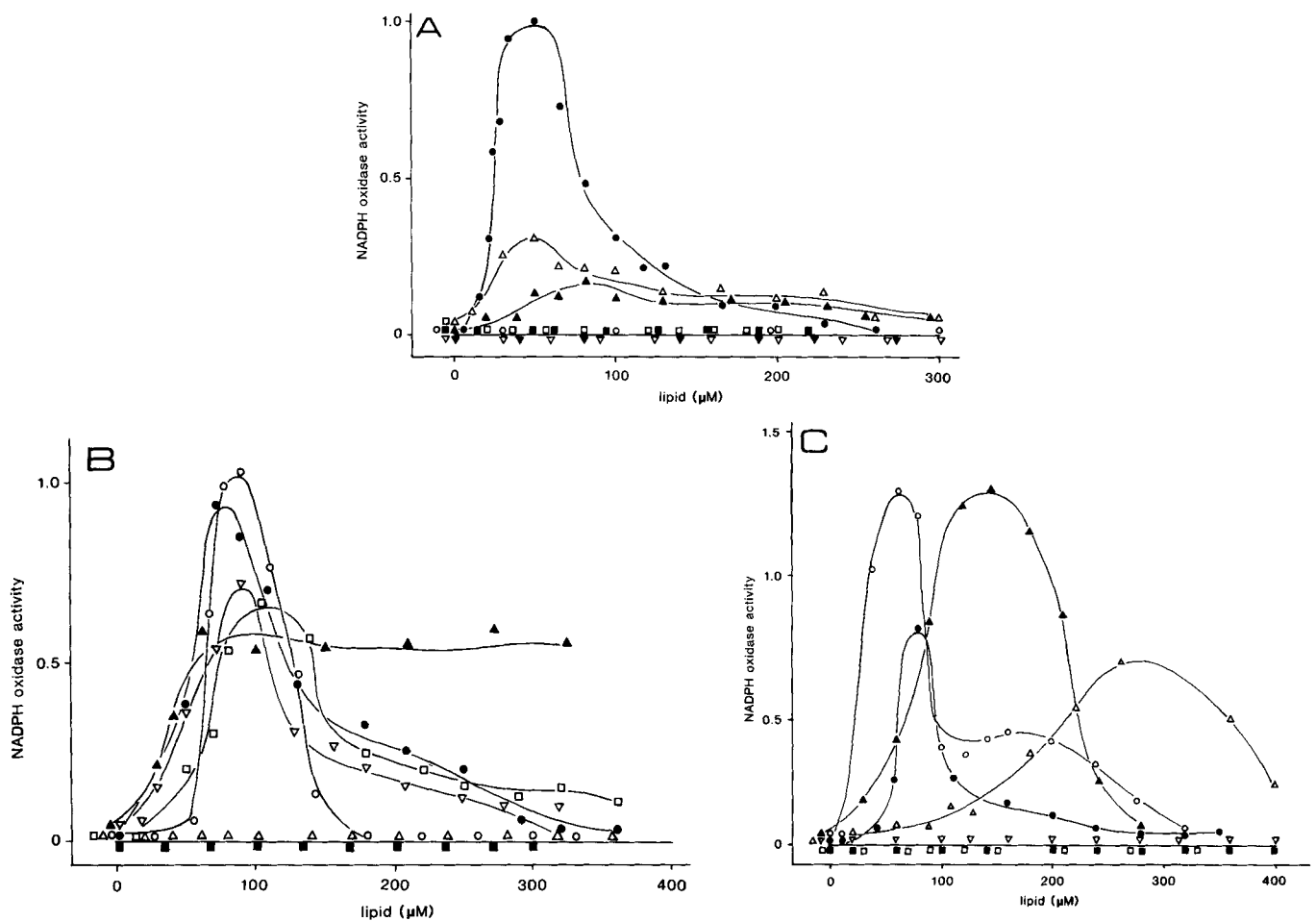


Fig. 2. Stimulation of NADPH oxidase in neutrophil plasma membranes by fatty acids, esters and SDS. Each set of experiments presented in one panel was performed with one membrane preparation. To allow comparison of the results presented in the three panels, which were obtained with different membrane preparations, NADPH oxidase activity is referred to by the maximal enzyme activity measured with arachidonic acid (50  $\mu\text{M}$ ), which varied between  $0.2\text{--}2\ \mu\text{mol O}_2^- \times \text{mg}^{-1} \times \text{min}^{-1}$ . Each point represents the mean of three or four experiments. (A) Arachidonic acid ( $\square$ ), homo- $\gamma$ -linolenic acid ( $\Delta$ ), arachidonic acid ( $\bullet$ ), methyl arachidonate ( $\blacksquare$ ), ETYA ( $\circ$ ), timnodonic acid ( $\blacktriangle$ ), erucic acid ( $\nabla$ ), nervonic acid ( $\blacktriangledown$ ). Assay mixtures contained 2  $\mu\text{g}$  plasma membrane protein and 30  $\mu\text{g}$  cytosolic protein. NADPH oxidase activity is expressed in relative units and is referred to by the enzyme activity measured with 50  $\mu\text{M}$  arachidonic acid, which amounted to  $2\ \mu\text{mol O}_2^- \times \text{mg}^{-1} \times \text{min}^{-1}$ . (B) Stearic acid ( $\Delta$ ), oleic acid ( $\square$ ), methyl oleate ( $\blacksquare$ ), elaidic acid ( $\blacktriangle$ ), linoleic acid ( $\bullet$ ), linolelaidic acid ( $\nabla$ ),  $\gamma$ -linolenic acid ( $\circ$ ). Assay mixtures contained 7  $\mu\text{g}$  plasma membrane protein and 100  $\mu\text{g}$  cytosolic protein. Enzyme activity is referred to by the activity measured with 50  $\mu\text{M}$  arachidonic acid, which amounted to  $0.2\ \mu\text{mol O}_2^- \times \text{mg}^{-1} \times \text{min}^{-1}$ . (C) Palmitic acid ( $\square$ ), palmitoleic acid ( $\bullet$ ), palmitelaidic acid ( $\circ$ ), myristic acid ( $\blacksquare$ ), myristoleic acid ( $\Delta$ ), lauric acid ( $\nabla$ ), SDS ( $\blacktriangle$ ). Assay mixtures contained 10  $\mu\text{g}$  membrane protein and 130  $\mu\text{g}$  cytosolic protein. Enzyme activity is referred to by the activity measured with 50  $\mu\text{M}$  arachidonic acid, which amounted to  $1.5\ \mu\text{mol O}_2^- \times \text{mg}^{-1} \times \text{min}^{-1}$ .

500  $\mu\text{M}$  NADPH, 10  $\mu\text{M}$  FAD, 100  $\mu\text{M}$  ferricytochrome *c*, 3 mM  $\text{MgCl}_2$ , 400  $\mu\text{M}$  ATP and 50 mM triethanolamine/HCl, pH 7.0. The specific activity of NADPH oxidase varied by a factor of 5–10 between different plasma membrane preparations as was previously reported [16, 19]. However, the specific activity varied only to a small extent in experiments carried out with one and the same preparation. In intact neutrophils, the rates of superoxide formation elicited by fMet-Leu-Phe also varied by a factor of 5–10 with different donors (unpublished results). Therefore, variation of NADPH oxidase activity in neutrophil plasma membranes may reflect the interindividual heterogeneity of neutrophil cell populations [20]. The results presented in the figures and tables were obtained with one membrane preparation and were typical for at least three experiments with different membrane preparations.  $\text{Me}_2\text{SO}$  up to 1.0% (v/v) and ethanol up to 2% (v/v) were without effect on NADPH oxidase activity.

Neutrophil cytosol (10 ml) was dialyzed for 48 h in four 2-l portions of dialysis buffer composed of 100 mM KCl, 3 mM NaCl, 1 mM EDTA, 10 mM Pipes, pH 7.3 at 4°C, using type 20/32 Visking dialysis tubing (Serva, Heidelberg, FRG) with an exclusion limit of 8–15 kDa. Protein determination was performed according to Lowry et al. [21]. NADPH, FAD, GTP[ $\gamma\text{S}$ ] and yeast hexokinase were purchased from Boehringer Mannheim (Mannheim, FRG). Ferricytochrome *c* (type III), superoxide dismutase, soybean lipoxygenase, H-7, saponin, Lubrol PX, fatty acids (99% pure) and esters (99% pure) were obtained from Sigma (Taufkirchen, FRG). SDS, Triton X-100 and sodium cholate were from Serva (Heidelberg, FRG). Bis(*tert*-butyl) peroxide was from Fluka (Buchs, Switzerland). ETYA was a gift of Hoffmann-La Roche (Basel, Switzerland). 4 $\beta$ -Phorbol 12-myristate 13-acetate was provided by Dr E. Hecker, Deutsches Krebsforschungszentrum (Heidelberg, FRG). Purified protein kinase C from chicken stomach with a specific activity of 25 nmol phosphate transferred  $\times \text{mg}^{-1} \times \text{min}^{-1}$  as assayed by incorporation of  $^{32}\text{P}$  into histone H-1 from [ $\gamma$ - $^{32}\text{P}$ ]ATP as described [6], was a gift of Dr C. Schächtele, Goedecke (Freiburg, FRG). All other chemicals and reagents were of analytical grade. H-7 (10 mM) was dissolved in  $\text{Me}_2\text{SO}$ . Stock solutions (10–20 mM) of fatty acids and esters were prepared in ethanol under a nitrogen atmosphere and light protection and stored at –20°C.

## RESULTS

Activation of superoxide formation in neutrophil plasma membranes depended on the addition of neutrophil cytosol as well as of arachidonic acid. In the absence of neutrophil cytosol, arachidonic acid did not activate NADPH oxidase. Stimulation of superoxide formation reached a plateau with a 9–16-fold excess of cytosolic protein in relation to plasma membrane protein (Fig. 1), depending on the preparation analyzed. Unless stated otherwise, all experiments were carried out with a maximally activating amount of cytosolic protein. No superoxide formation was detectable in the absence of arachidonic acid as was previously shown [16]. Cytosol which was heated for 10 min at 95°C no longer constituted NADPH oxidase activity. Cytosols from murine P 388 D<sub>1</sub> monocyte macrophages, rat liver, kidney or brain did not substitute neutrophil cytosol (data not shown). These results indicate that the cytosolic cofactor is heat-labile and apparently specific for neutrophils.

As it is not known whether activation of NADPH oxidase in cell-free systems from human neutrophils is specific for

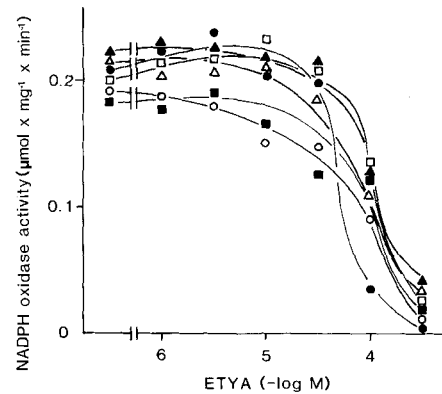


Fig. 3. Inhibition of NADPH oxidase in neutrophil plasma membranes by ETYA. 2 min prior to the addition of unsaturated fatty acids, ETYA at the concentrations indicated was added to reaction mixtures containing 10  $\mu\text{g}$  plasma membrane protein and 160  $\mu\text{g}$  cytosolic protein. (□) Oleic acid (110  $\mu\text{M}$ ), (■) linoleic acid (60  $\mu\text{M}$ ), (○)  $\gamma$ -linolenic acid (60  $\mu\text{M}$ ), (●) arachidonic acid (30  $\mu\text{M}$ ), (▲) palmitelaidic acid (100  $\mu\text{M}$ ), (△) linolelaidic acid (90  $\mu\text{M}$ ). Data represent the mean of three or four experiments

arachidonic acid [12, 13], the ability of various fatty acids, esters and detergents to activate NADPH oxidase in neutrophil plasma membranes was examined (Fig. 2). *Cis*- and *trans*-unsaturated fatty acids with chain lengths of up to 20 carbon atoms activated NADPH oxidase. The maximum superoxide generation rates were in the same order of magnitude as the rate obtained with a maximally effective concentration of arachidonic acid (50  $\mu\text{M}$ ). Activation by most fatty acids was biphasic with maximum effects at concentrations between 50  $\mu\text{M}$  and 260  $\mu\text{M}$ . Saturated fatty acids, the acetylenic analogue of arachidonic acid, ETYA, and esters of unsaturated fatty acids were inactive, indicating that both a double bond and the unesterified carboxyl group are essential for NADPH oxidase activation. SDS was also a potent activator of NADPH oxidase (see Fig. 2). Other detergents such as Lubrol PX, Triton X-100 and sodium cholate (0.001–0.1%, w/v) as well as saponin (0.5–500  $\mu\text{g}/\text{ml}$ ) were inactive (data not shown). The lipid concentrations necessary to activate NADPH oxidase maximally and the dose/response curves were very similar in different membrane preparations and at varying relations between plasma membrane protein and cytosolic protein (see Figs 2A and 5). NADPH oxidase activation was temperature-dependent and reached a maximum at 10°C for palmitelaidic acid, at 20°C for palmitoleic and linolelaidic acid and at 25°C for arachidonic acid and SDS (data not shown). There was no correlation between the number of double bonds, their *cis* or *trans* configuration, the melting points [22], hydrophobicity [23–25] and critical micelle concentration of fatty acids [25–27], on the one hand, and the potency of fatty acids to induce superoxide generation and the temperature optimum on the other hand.

It has been suggested that lipoxygenase products of arachidonic acid serve as intermediates in the stimulation of NADPH oxidase [14, 15]. Therefore, the effect of ETYA, a potent inhibitor of lipoxygenases and cyclooxygenase [28, 29], on NADPH oxidase activation was studied (Fig. 3). ETYA at concentrations higher than 30  $\mu\text{M}$  inhibited superoxide formation elicited by *cis*-polyunsaturated fatty acids, which are substrates for lipoxygenases [30] and by *cis*-monounsaturated and *trans*-unsaturated fatty acids, both of which are not substrates for lipoxygenases [30, 31]. Hydrogen peroxide and bis(*tert*-butyl) peroxide (0.1–1000  $\mu\text{M}$ ) did not activate

Table 1. Effect of H-7 on NADPH oxidase activity in neutrophil plasma membranes stimulated with fatty acids and SDS 2 min prior to fatty acids or SDS, H-7 or solvent was added to assays containing 12  $\mu\text{g}$  plasma membrane protein and 110  $\mu\text{g}$  cytosolic protein. Data represent the mean  $\pm$  SEM of five experiments

Lipid	Concn $\mu\text{M}$	NADPH oxidase activity with H-7 at			
		0	1 $\mu\text{M}$	10 $\mu\text{M}$	100 $\mu\text{M}$
		$\mu\text{mol} \times \text{mg}^{-1} \times \text{min}^{-1}$			
Arachidonic acid	50	$0.13 \pm 0.03$	$0.14 \pm 0.01$	$0.13 \pm 0.02$	$0.11 \pm 0.03$
Palmitelaic acid	60	$0.08 \pm 0.01$	$0.09 \pm 0.02$	$0.07 \pm 0.01$	$0.06 \pm 0.01$
SDS	150	$0.20 \pm 0.01$	$0.20 \pm 0.01$	$0.20 \pm 0.01$	$0.19 \pm 0.01$

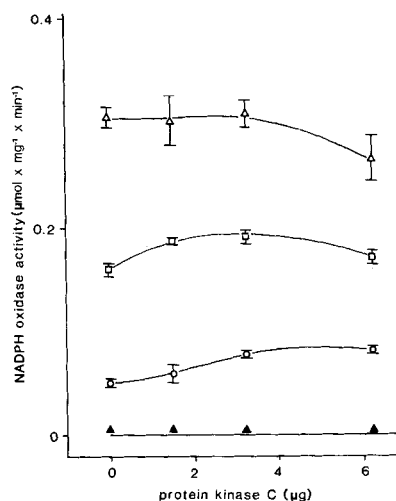


Fig. 4. Influence of purified protein kinase C on NADPH oxidase activity in neutrophil plasma membranes in the presence of different amounts of neutrophil cytosolic protein. Assays contained 10  $\mu\text{g}$  plasma membrane protein and the following amounts of neutrophil cytosolic protein: 0 ( $\blacktriangle$ ), 40  $\mu\text{g}$  ( $\circ$ ), 80  $\mu\text{g}$  ( $\square$ ), 160  $\mu\text{g}$  ( $\triangle$ ). Protein kinase C was added at the amounts indicated on the abscissa. Reaction mixtures contained 3 mM  $\text{MgCl}_2$  and 400  $\mu\text{M}$  ATP. Reactions were initiated with arachidonic acid (50  $\mu\text{M}$ ). Data represent the mean  $\pm$  SEM of three experiments

NADPH oxidase. Soybean lipoxygenase was no substitute for neutrophil cytosol (data not shown).

Arachidonic acid activates protein kinase C [32–34]. To assess the role of protein kinase C in NADPH oxidase activation, the effect of H-7, a potent inhibitor of protein kinase C [7], on superoxide formation was examined (Table 1). Superoxide formation elicited by fatty acids and SDS was not inhibited by H-7 at concentrations of up to 100  $\mu\text{M}$ . 4 $\beta$ -phorbol 12-myristate 13-acetate, a potent activator of protein kinase C, at concentrations of up to 100 ng/ml did not activate superoxide generation in neutrophil plasma membranes (data not shown). Protein kinase C, purified from chicken stomach, was no substitute for neutrophil cytosol and did not enhance superoxide formation in the presence of neutrophil cytosol (Fig. 4). Removal of cytosolic ATP by preincubation of assay mixtures with hexokinase and glucose reduced the stimulatory effect of arachidonic acid by 50% and enhanced the stimulatory activity of GTP[ $\gamma$ S] up to fivefold (Table 2). To exclude a role of calcium in the activation process, reaction mixtures were preincubated with EGTA (10 mM). Chelation of calcium by EGTA did not inhibit the stimulatory effects of arachidonic acid and GTP[ $\gamma$ S] (Table 3). Dialyzed neutrophil cytosol reconstituted NADPH oxidase stimulation by

Table 2. Effect of removal of ATP by hexokinase and glucose on stimulation of NADPH oxidase by arachidonic acid and GTP[ $\gamma$ S] in neutrophil plasma membranes

Assays contained 10  $\mu\text{g}$  plasma membrane protein and 160  $\mu\text{g}$  cytosolic protein and 0.2  $\mu\text{mol}$  ATP. Assay mixtures were preincubated for 8 min at 27.5°C with 10 mM glucose and 7 U hexokinase to remove cytosolic ATP. Controls were preincubated with solvent. Reactions were initiated with arachidonic acid (50  $\mu\text{M}$ ). Data represent the mean  $\pm$  SEM of four experiments

Addition	NADPH oxidase activity	
	control	+ GTP[ $\gamma$ S] (10 $\mu\text{M}$ )
	$\mu\text{mol} \times \text{mg}^{-1} \times \text{min}^{-1}$	
None	$0.43 \pm 0.03$	$1.40 \pm 0.12$
Glucose	$0.56 \pm 0.06$	$1.45 \pm 0.03$
Hexokinase	$0.34 \pm 0.04$	$0.95 \pm 0.07$
Glucose + hexokinase	$0.16 \pm 0.01$	$0.84 \pm 0.10$

Table 3. Effect of dialysis of neutrophil cytosol and chelation of calcium with EGTA on stimulation of NADPH oxidase by arachidonic acid and GTP[ $\gamma$ S] in neutrophil plasma membranes

Neutrophil plasma membranes (150  $\mu\text{g}$  protein) were washed three times in 1.5 ml 50 mM triethanolamine/HCl, pH 7.0, sedimented by centrifugation at 10000  $\times g$  for 10 min at 4°C and resuspended in 1 ml 50 mM triethanolamine/HCl, pH 7.0. Assays contained 4  $\mu\text{g}$  plasma membrane protein, 50  $\mu\text{g}$  non-dialyzed cytosolic protein and 200  $\mu\text{g}$  dialyzed cytosolic protein, respectively. 2 min prior to arachidonic acid (50  $\mu\text{M}$ ). GTP[ $\gamma$ S] and/or EGTA was added to reaction mixtures. Data represent the mean  $\pm$  SEM of four or five experiments

Cytosol	EGTA mM	NADPH oxidase activity	
		control	+ GTP[ $\gamma$ S] (10 $\mu\text{M}$ )
		$\mu\text{mol} \times \text{mg}^{-1} \times \text{min}^{-1}$	
Non-dialyzed	0	$0.26 \pm 0.03$	$0.76 \pm 0.03$
Non-dialyzed	10	$0.22 \pm 0.02$	$0.59 \pm 0.03$
Dialyzed	0	$0.17 \pm 0.02$	$0.49 \pm 0.06$
Dialyzed	10	$0.14 \pm 0.03$	$0.51 \pm 0.04$

arachidonic acid and GTP[ $\gamma$ S] in neutrophil plasma membranes in the absence or presence of EGTA (see Table 3). These findings suggest that low-molecular-mass compounds including ATP, magnesium and calcium, were not essential for NADPH oxidase activation.

The stimulatory effect of GTP[ $\gamma$ S] was observed with various amounts of neutrophil cytosolic protein and with

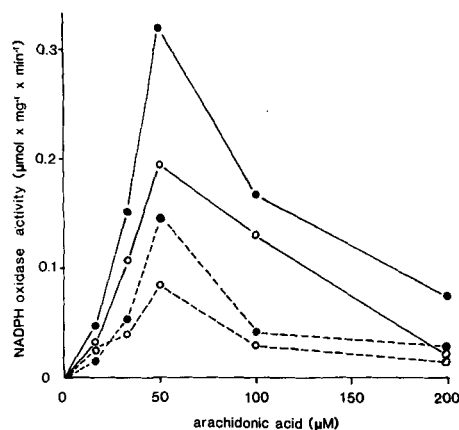


Fig. 5. Stimulation of NADPH oxidase in neutrophil plasma membranes by neutrophil cytosol, arachidonic acid and GTP[ $\gamma$ S]. Reaction mixtures contained 10  $\mu$ g plasma membrane protein, (○) half-maximally activating amount of cytosolic protein (60  $\mu$ g) or (●) a maximally activating amount of cytosolic protein (160  $\mu$ g). GTP[ $\gamma$ S] (—) or solvent (---) was added to the assay mixtures 2 min prior to arachidonic acid. Data represent the mean of three experiments

Table 4. Stimulation of NADPH oxidase activity in neutrophil plasma membranes by GTP[ $\gamma$ S] with fatty acids and SDS

2 min prior to addition of fatty acids or SDS, GTP[ $\gamma$ S] or solvent was added to the assay mixture containing 12  $\mu$ g plasma membrane protein and 110  $\mu$ g cytosolic protein. Data represent the mean  $\pm$  SEM of three experiments

Lipid	Concn $\mu$ M	NADPH oxidase activity	
		control	+ GTP[ $\gamma$ S] (10 $\mu$ M)
	$\mu$ M	$\mu$ mol $\times$ mg $^{-1}$ $\times$ min $^{-1}$	
Homo- $\gamma$ -linolenic acid	65	0.05 $\pm$ 0.01	0.12 $\pm$ 0.01
Arachidonic acid	50	0.13 $\pm$ 0.02	0.42 $\pm$ 0.06
Timnodonic acid	100	0.03 $\pm$ 0.01	0.07 $\pm$ 0.01
Linolelaidic acid	60	0.07 $\pm$ 0.01	0.20 $\pm$ 0.01
Palmitoleic acid	80	0.05 $\pm$ 0.01	0.14 $\pm$ 0.01
Palmitelaidic acid	60	0.10 $\pm$ 0.01	0.24 $\pm$ 0.03
SDS	150	0.20 $\pm$ 0.01	0.23 $\pm$ 0.01

arachidonic acid at different concentrations (Fig. 5). In the presence of a maximally activating amount of cytosolic protein, GTP[ $\gamma$ S] (10  $\mu$ M) enhanced superoxide generation 2–4-fold with arachidonic acid at concentrations between 16–200  $\mu$ M. With a half-maximally activating amount of cytosolic protein, the stimulatory effect of GTP[ $\gamma$ S] was observed only with arachidonic acid at concentrations between 30–100  $\mu$ M. GTP[ $\gamma$ S] (10  $\mu$ M) also enhanced superoxide formation up to threefold in the presence of other *cis*- and *trans*-unsaturated fatty acids but not in the presence of SDS, indicating that the effect of GTP[ $\gamma$ S] was specific for unsaturated fatty acids (Table 4).

## DISCUSSION

Stimulation of NADPH oxidase in neutrophil plasma membranes requires two cofactors in order to exhibit catalytic activity, i.e. neutrophil cytosol and fatty acids. Several mechanisms for activation of NADPH oxidase by fatty acids

have been suggested, amongst others activation by lipoxygenase products of arachidonic acid [14, 15],

Unsaturated fatty acids activated superoxide formation in neutrophil plasma membranes, regardless of whether they are or are not substrates for lipoxygenases. ETYA inhibited superoxide formation in neutrophil plasma membranes elicited by fatty acids at concentrations higher than 30  $\mu$ M, i.e. concentrations, which are higher than those required for inhibition of lipoxygenases [29]. Soybean lipoxygenase was no substitute for neutrophil cytosol, and peroxides did not activate NADPH oxidase in neutrophil plasma membranes. Thus, it appears that hydroperoxides of unsaturated fatty acids are not obligatory intermediates in activation of superoxide formation in plasma membranes of human neutrophils. The inhibitory effect of ETYA on superoxide formation in neutrophil plasma membranes may be explained by competitive antagonism of ETYA and unsaturated fatty acids at sites which are not localized on lipoxygenases. There is recent evidence that, in intact neutrophils [35] and macrophages [36], lipoxygenase metabolites of arachidonic acid are not involved in NADPH oxidase activation.

It has been suggested that the stimulatory effect of arachidonic acid on NADPH oxidase is mediated by activation of protein kinase C which may also represent the cytosolic cofactor [4, 12, 33]. Our results do not support this hypothesis: H-7 inhibits protein kinase C with a  $K_i$  value of 6  $\mu$ M [7] but, at concentrations of up to 100  $\mu$ M, H-7 did not inhibit superoxide generation in neutrophil plasma membranes. In intact neutrophils, H-7 did not inhibit arachidonic-acid-induced superoxide formation [35]. Only *cis*-unsaturated fatty acids but not *trans*-unsaturated fatty acids activate protein kinase C [34]. The concentrations of fatty acids required for maximum activation of protein kinase C are higher [32–34] than those required for maximum activation of NADPH oxidase in neutrophil plasma membranes. In addition, removal of cytosolic ATP as substrate for protein kinase C by hexokinase and glucose or by dialysis of neutrophil cytosol as well as chelation of calcium by EGTA did not abolish NADPH oxidase activation by arachidonic acid. Finally, purified protein kinase C was not a substitute for neutrophil cytosol. Therefore, it is unlikely that fatty acids mediate activation of NADPH oxidase by stimulation of protein kinase C. 4 $\beta$ -Phorbol myristate 13-acetate did not induce superoxide formation in neutrophil plasma membranes as is the case with lysates of mouse macrophages [36]. In contrast, it was shown by Cox et al. [6] that 4 $\beta$ -phorbol 12-myristate 13-acetate in the presence of neutrophil cytosol, activated NADPH oxidase in neutrophil membranes, which was suppressed by removal of ATP, magnesium and chelation of calcium. In addition, purified protein kinase C from rat brain was a substitute for neutrophil cytosol.

In intact neutrophils, only *cis*-unsaturated fatty acids induce superoxide formation, whereas *trans*-unsaturated and saturated fatty acids are inactive. The potencies of fatty acids to induce superoxide formation correlate with physico-chemical parameters, i.e. melting points of fatty acids and increase in membrane fluidity [10, 11, 37, 38]. In contrast, in neutrophil plasma membranes *trans*-unsaturated fatty acids were as potent activators of NADPH oxidase as were *cis*-unsaturated fatty acids. There was no correlation between concentrations of fatty acids to activate NADPH oxidase maximally, the maximum rates of superoxide formation or temperature optimum and melting points [22] or hydrophobicity of fatty acids [23–25]. Thus, physico-chemical properties of fatty acids alone do not provide a sufficient

basis to explain their different NADPH-oxidase-activating potencies. Further investigations will have to be performed to elucidate whether binding of individual fatty acids to specific neutrophil cytosolic or plasma membrane proteins may account for the differences observed with unsaturated fatty acids. All fatty acids and SDS induced superoxide generation at concentrations below their critical micelle concentrations [25–27], indicating that the monomeric species but not the micellar forms mediate activation of NADPH oxidase. The concentration of arachidonic acid that induced maximal superoxide generation rates is higher than that obtainable within the cell under physiological conditions (1–10  $\mu\text{M}$ ) [36]. However, we recently reported that arachidonic acid at a concentration of 16  $\mu\text{M}$  induced superoxide generation in neutrophil plasma membranes [16]. Most other fatty acids at these concentrations did not substantially activate NADPH oxidase. In mouse macrophages, the release of arachidonic acid is no prerequisite for the activation of superoxide formation [36]. Thus, the question remains open as to whether arachidonic acid may act as second messenger to trigger activation of NADPH oxidase in the intact cell [14, 15].

Extending our recent observations that arachidonic-acid-induced superoxide formation in neutrophil plasma membranes is potentiated by GTP[ $\gamma$ S] [16], we report here that the stimulatory effect of GTP[ $\gamma$ S] is found in the presence of submaximally and maximally activating amounts of neutrophil cytosolic protein and with arachidonic acid at various concentrations. Superoxide formation elicited by other unsaturated fatty acids was also enhanced up to threefold by GTP[ $\gamma$ S]. The failure of SDS to enhance NADPH oxidase activity substantially with GTP[ $\gamma$ S] may be due to denaturation of a protein component essential for the activation process. Enzyme stimulation by GTP[ $\gamma$ S] was abolished neither by depletion of cytosolic ATP by hexokinase and glucose nor by dialysis of cytosol nor by chelation of calcium by EGTA. As a stimulatory effect of GTP[ $\gamma$ S] on protein kinase C in plasma membranes of rabbit neutrophils was only observed in the presence but not in the absence of calcium [39], it is unlikely that the stimulatory effect of GTP[ $\gamma$ S] on NADPH oxidase is mediated by protein kinase C. These results support our recent suggestion that NADPH oxidase represents an N-protein-regulated effector system [16]. Besides the chemotactic peptide, fMet-Leu-Phe, a variety of agonists, such as anaphylatoxin C5a [40], IgG [41], leukotriene B<sub>4</sub> [42], platelet-activating factor [41, 42] and opioid peptides [43] bind to plasma membrane receptors and activate NADPH oxidase. Recently, Volpi et al. [44] demonstrated that activation of neutrophils by leukotriene B<sub>4</sub> is not mediated by polyphosphoinositide breakdown. Therefore, it remains to be clarified whether NADPH oxidase is regulated by the same N-protein (N<sub>p</sub>) that has been suggested to be involved in fMet-Leu-Phe-induced activation of phospholipase C [45, 46], N<sub>i</sub>, the N-protein involved in the inhibition of adenylate cyclase [17], the putative N-protein (N<sub>e</sub>) assumed by Barrowman et al. to be involved in the exocytotic mechanism of secretion [47] or by a distinct N-protein specific for NADPH oxidase.

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