Deactivation of neutrophil NADPH oxidase by actin-depolymerizing agents in a cell-free system

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The cell-free activation of human neutrophil NADPH oxidase (O_2^- -generating enzyme) is enhanced by actin [Morimatsu, Kawagoshi, Yoshida and Tamura (1997) *Biochem. Biophys. Res. Commun.* **230**, 206–210]. In an attempt to elucidate the mechanism, we examined the effect of actin-depolymerizing agents on the duration of NADPH oxidase in a cell-free system. The addition of DNase I, an F-actin-depolymerizing protein, caused an accelerated deactivation of the oxidase. The deactivation was also facilitated by latrunculin A, a sponge toxin that depolymerizes F-actin. Exogenously added actin prevented the deactivation by DNase I or latrunculin A, whereas EDTA accelerated a dilution-induced deactivation of the oxidase and

Mg^{2+} ions retarded it. The stability in dilution was found to correlate well with free Mg^{2+} concentration. Estimation of F-actin in the system showed that F-actin increased during the oxidase activation and that DNase I or EDTA decreased F-actin content in parallel with the activity. Treatment of the cell-free mixture with a chemical cross-linker prevented the deactivation and F-actin decrease by EDTA. Taken together, these results suggest that actin filaments which grow during the activation of NADPH oxidase prolong the lifetime of the oxidase.

Key words: cytoskeleton, F-actin, magnesium ion, phagocytosis, superoxide.

INTRODUCTION

Superoxide (O_2^-) generation by neutrophils has an important role in host defence against microbial infection [1]. The enzyme responsible for O_2^- generation is called NADPH oxidase (or respiratory burst oxidase), which is dormant in resting cells and becomes active upon cell activation [2]. The oxidase can also be activated in a cell-free system including plasma membrane and cytosol in the presence of an anionic amphiphile [2]. The oxidase consists of membrane cytochrome b_{558} (gp91^{phox} and p22^{phox}) and cytosolic proteins (p47^{phox}, p67^{phox} and Rac) [3], which assemble into an active complex upon activation. Recently p40^{phox}, which is bound to p67^{phox}, has been suggested as a regulator [3]. The oxidase is extremely labile when activated either *in vivo* [4] or *in vitro* [5] and the deactivation is assumed to involve the dissociation of a cytosolic component or components from cytochrome b_{558} .

Actin, a major component of cytoskeleton, is abundantly present in neutrophils [6]. Approx. one-third of the total actin is present in polymeric form (F-actin) in resting neutrophils [7] and upon cell activation additional monomer actin (G-actin) polymerizes into F-actin. Actin polymerization has long been related to neutrophil chemotaxis or phagocytosis [6], probably because they involve motion and shape change. However, the relation to O_2^- generation has not been considered.

Quinn et al. [8] reported that NADPH oxidase activity from phorbol ester-stimulated neutrophils co-sediments with heavy plasma-membrane fraction that includes actin and fodrin. Woodman et al. [9] found that the activated oxidase is restricted to the membrane cytoskeleton. We showed previously that the labile enzyme is remarkably stabilized by chemical cross-linking [4,5]. However, the stabilized activity cannot be extracted, even by Triton, suggesting that the enzyme complex is linked to the cytoskeleton.

Recently we found that actin, either endogenous or exogenous, enhances the activation of the oxidase in a cell-free system [10]. In the present study, to elucidate the mechanism for the phenomenon we examined several F-actin-depolymerizing agents on the stability of the oxidase. We found that actin-depolymerizing agents markedly facilitated the deactivation of NADPH oxidase, and suggested a role of F-actin to prolong the lifetime of NADPH oxidase.

EXPERIMENTAL

Materials

DNase I (molecular-biology grade) was obtained from Worthington Biochemical Corp. (Lakewood, NJ, U.S.A.). Actin (rabbit skeletal muscle, AKL95) was purchased from Cytoskeleton (Denver, CO, U.S.A.). Latrunculin A was obtained from Wako Pure Chemicals (Osaka, Japan). E64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] was a product of Peptide Institute (Osaka, Japan). Phalloidin, tetramethylrhodamine isothiocyanate (TRITC)-phalloidin and guanosine 5'-[γ -thio]triphosphate (GTP[S]) were purchased from Sigma Aldrich (St. Louis, MO, U.S.A.). All other reagents were of the best grade commercially available.

Neutrophil preparation and subcellular fractionation

Isolation of human neutrophils and subcellular fractionation were carried out as described previously [5]. The isolated cells were suspended in buffer A [100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1 mM ATP, 10 mM potassium phosphate buffer (KPB), pH 7.3] and subjected to nitrogen cavitation. After sucrose-gradient separation, the plasma membrane fraction was diluted with 1 vol. of buffer B (100 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$,

Abbreviations used: E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane; KPB, potassium phosphate buffer; TRITC, tetramethylrhodamine isothiocyanate; GTP[S], guanosine 5'-[γ -thio]triphosphate; EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide.

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1 mM EGTA, 10 mM Pipes, pH 7.0), sedimented by centrifugation at 200 000 *g* for 1 h, and resuspended in modified buffer B (with all the concentrations given above halved) containing 0.34 M sucrose.

Cell-free activation and assay for O_2^- generation

The standard conditions for assay were as follows. Plasma membrane (15 μ g of protein, 5×10^6 cell equivalents) and cytosol (210 μ g of protein, 9×10^6 cell equivalents) were mixed per 100 μ l in 20 mM KPB (pH 7.0), supplemented with SDS (final concentration 240 μ M), and incubated for 10 min at 25 °C. An aliquot (10 μ l) of the mixture was mixed with 240 μ l of 20 mM KPB (pH 7.0) containing 80 μ M cytochrome *c* and 200 μ M NADPH, and the mixture (120 μ l each) was transferred into two cuvettes, one of which (reference) contained superoxide dismutase (80 μ g/ml). O_2^- -generating activity was assayed at 25 °C as described previously [11]. O_2^- -generating activities were expressed as nmol of O_2^- formed/min per mg of plasma-membrane protein.

Pretreatment of DNase I

Because DNase I prepared from pancreas contains trace amounts of chymotrypsin and other proteases, and because NADPH oxidase activity is inhibited by some protease inhibitors at high concentrations, we treated DNase I with irreversible inhibitors prior to the experiments and then removed unreacted reagents by dialysis. DNase I preparation was incubated with 500 μ M diisopropyl fluorophosphate, 500 μ M PMSF and 10 μ M E64 at 0 °C for 20 min and then dialysed against 20 mM KPB (pH 7.0) for 4 h at 0 °C.

Measurement of F-actin content

F-actin content was measured following the method of Redmond et al. [12] with modifications. The cell-free activated mixture containing plasma membrane (15 μ g) and cytosol (210 μ g) per 100 μ l was fixed with 1% glutaraldehyde at 0 °C for 1 h and treated with 0.2 μ M TRITC-phalloidin with or without 2 μ M phalloidin at 0 °C for 1 h. The mixture was centrifuged at 340 000 *g* at 4 °C for 15 min with a compact ultracentrifuge (Hitachi CS150GX), and the pellets were extracted with 1 ml of methanol for 48 h. The supernatant was obtained by centrifugation at 2000 *g* at 4 °C for 5 min. The fluorescence of the supernatant was measured with a spectrofluorimeter (Shimadzu RF-5300 PC; excitation at 540 nm and emission at 565 nm). The difference in fluorescence intensity between TRITC-phalloidin-treated sample and phalloidin + TRITC-phalloidin-treated sample was calculated and expressed as Δ RFI (relative fluorescence intensity), which reflects the specific binding of phalloidin to F-actin.

Dilution experiments

In dilution experiments, the activated mixture was diluted with 10 mM Pipes (pH 7.0) alone or the same buffer containing EDTA and/or $MgCl_2$ and incubated at 0 °C. In some experiments, EDTA and $MgCl_2$ were included in the dilution buffer to afford an indicated free Mg^{2+} concentration. After the incubation, the mixture was supplemented with cytochrome *c* and NADPH, warmed up to 25 °C for 30 s, and assayed for O_2^- generation without further dilution. The standard cell-free system contained 0.2 mM ATP and 0.8 mM $MgCl_2$ brought from cytosol and plasma membrane preparations.

Calculation of free Mg^{2+} concentration

Free Mg^{2+} concentration [Mg^{2+}] in the system was calculated using the equation described by Sunyer et al. [13]:

$$[Mg^{2+}]_t = [Mg^{2+}]\{1 + [EDTA]_t/([Mg^{2+}] + K_{EDTA}) + [ATP]_t/([Mg^{2+}] + K_{ATP}) + [GTP[S]]_t/([Mg^{2+}] + K_{GTP[S]})\}$$

where [Mg^{2+}]_t, [EDTA]_t, [ATP]_t and [GTP[S]]_t are the total concentrations of those compounds. K_{EDTA} , K_{ATP} , and $K_{GTP[S]}$ are the dissociation constants (K_d) of the Mg^{2+} -EDTA (0.4 μ M) [13], Mg^{2+} -ATP (40 μ M) [14] and Mg^{2+} -GTP[S] (60 μ M) [13] complexes, respectively.

RESULTS

Effect of DNase I

DNase I is known to depolymerize F-actin by binding G-actin tightly [15] and shifting the equilibrium from F- to G-actin [16]. Therefore, we examined the effect of DNase I on the stability of NADPH oxidase activity. As shown in Figure 1(A), DNase I markedly accelerated the deactivation of the oxidase at 4 °C. The activity fell to 54% in the first 10 min and then gradually decreased. In a first-order plot of the data, the deactivation was biphasic (results not shown) and the half-life ($t_{1/2}$) for the first phase was 8 min (Table 1), which was much shorter than that for the control (160 min). In contrast, the second-phase decay ($t_{1/2}$ = 165 min) was similar to that for control. The deactivation by DNase I was prevented by addition of actin (100 μ M). When actin was added to the mixture by itself, the oxidase activity was stabilized further (Figure 1A). These results suggest that actin filaments play a role in maintaining the oxidase activity, although some subpopulation of the oxidase seems unrelated to actin filaments.

Figure 1(B) shows the concentration dependence for DNase I in the deactivation of NADPH oxidase. The deactivation by DNase I occurred in a dose-dependent manner, and when DNase I was used at 2.5 mg/ml, the activity was decreased to 47%. At higher concentrations the activity was not much changed (results not shown). The addition of actin (100 μ M) completely prevented the deactivation by DNase I.

The prevention by actin of the DNase I-induced deactivation was examined in more detail. When different amounts of actin were used, the activity was restored in a concentration-dependent manner (Figure 1C), whereas ovalbumin showed no such effect, indicating that it was not a general protein effect (results not shown). Interestingly when F-actin, actin polymerized before addition, was added to the incubation mixture, a similar effect was obtained. Although F-actin does not inhibit DNase I, it could be a source of G-actin when endogenous G-actin is captured by DNase I, protecting endogenous F-actin from depolymerization. These results indicate that F-actin has an ability to sustain the oxidase activity.

Effect of latrunculin

To ascertain a role of F-actin, we examined another F-actin-depolymerizing agent. Latrunculin A, a marine toxin, is known to depolymerize F-actin by a mechanism similar to DNase I. Figure 2 shows the time course of the oxidase activity in the incubation with or without latrunculin A. Latrunculin apparently accelerated the deactivation, and at 30 min the activity was 55% of the control. The first-order plot of the deactivation curve was biphasic and the $t_{1/2}$ value for the first phase was estimated to be 13 min (Table 1), which is much less than that for the control

Table 1 Half-lives of O_2^- -generating activity in the cell-free activation mixture under various conditions

Half-lives ($t_{1/2}$) were estimated from a first-order plot of the data described in Figures 1(A), 2 and 4 using linear least-squares regression analysis. Incubation conditions not specified are provided in each Figure legend. The values in parentheses are the half-lives for the 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC)-treated sample described in Table 3 (see below).

Temperature	Conditions	$t_{1/2}$ (min)	
		First phase	Second phase
4 °C	None	160	—
	DNase I	8	165
	DNase I + actin	180	—
0 °C	None (DMSO)	310	—
	Latrunculin	13	300
	Latrunculin + actin	460	—
	Dilution	12	200
	Dilution + EDTA	4 (190)	60
	Dilution + Mg^{2+}	26 (> 350)	320

(310 min). The $t_{1/2}$ value for the second phase (300 min) was similar to that for the control. The deactivation by latrunculin was prevented by addition of actin in a dose-dependent manner and at 100 μ M actin completely prevented the deactivation by latrunculin (results not shown).

F-actin content

To confirm that F-actin was actually decreased by DNase I or latrunculin A, F-actin content was measured with fluorescently labelled phalloidin, which selectively binds to F-actin. As shown in Table 2, either DNase I or latrunculin decreased F-actin content in the system. F-actin levels were 56 and 63 % of the control after the incubation with DNase I and latrunculin, respectively. The result supports the concept that actin depolymerization deactivates the oxidase.

In the course of the experiment, we noticed that F-actin level increased in the cell-free activation with SDS. Therefore we next examined the relationship between the activation and F-actin level. F-actin level was measured after incubation with or without SDS (Figure 3). The incubation with SDS resulted in a 70 % increase in F-actin level. When the activation was performed with sub-optimal concentrations of SDS, F-actin level increased almost in parallel with the oxidase activity over a threshold of

Figure 1 The deactivation of NADPH oxidase in the presence and absence of DNase I and/or actin

(A) Time course. After cell-free activation, the mixture was incubated at 4 °C for 0–60 min in the absence (●) or presence of 2.5 mg/ml DNase I (△), 2.5 mg/ml DNase I + 100 μ M actin (○) and 100 μ M actin (□) in a total of 75 μ l. After incubation, an aliquot of the mixture was assayed for O_2^- generation as described in the Experimental section. Data are expressed as means $\pm$ S.D. of three determinations. The initial activities of the control and the samples with DNase I, DNase I + actin and actin were 952 ± 9.2 , 904.8 ± 17.5 , 943.8 ± 18.9 and 1079.4 ± 12.8 nmol/min per mg of plasma-membrane protein, respectively. (B) Concentration-dependence of DNase I. The cell-free activation mixture was incubated with various amounts of DNase I in the presence and absence of 100 μ M actin. The control activities (100%) with and without actin were 742.9 ± 15.1 and 651.6 ± 13.4 nmol/min per mg of plasma-membrane protein, respectively. (C) Effect of actin concentration on DNase I-induced deactivation. The activation mixture was incubated for 1 h with DNase I (2.5 mg/ml) plus 0–100 μ M actin in a total of 60 μ l. In some experiments, actin (100 μ M) was polymerized before addition by incubating in 8 mM $MgCl_2$ /20 mM KPB (pH 7.0) at 0 °C for 1 h (▲). The control activity at 60 min was 727 ± 10.5 nmol/min per mg of plasma-membrane protein.

Figure 2 Effect of latrunculin A on the time course of NADPH oxidase activity

After cell-free activation the mixture was incubated with DMSO (○), 50 μ M latrunculin (△) or 50 μ M latrunculin+100 μ M actin (□) at 0 °C for 0–60 min and assayed for O_2^- generation. Data are expressed as means $\pm$ S.D. of three determinations. The initial activity of the control was 994.1 ± 19.8 nmol/min per mg of plasma-membrane protein.

Figure 3 The oxidase activation and F-actin content using different concentrations of SDS

The cell-free activation was performed with 0, 80, 160 and 240 μ M SDS, and the mixture was assayed for O_2^- generation and subjected to F-actin measurement as described in the Experimental section. Data are expressed as means $\pm$ S.D. of three determinations. Δ RFI, relative fluorescence intensity.

Table 2 Effect of DNase I or latrunculin on NADPH oxidase and F-actin content

The cell-free activated mixture was incubated at 4 °C for 1 h in the presence and absence of DNase I (2.5 mg/ml) or incubated at 0 °C for 30 min in the presence and absence of latrunculin A (100 μ M). After the incubation, the mixture was assayed for O_2^- generation and subjected to F-actin estimation. The values in parentheses are the data as percentages relative to the respective controls (set at 100%). Δ RFI, relative fluorescence intensity.

Addition	O_2^- -generating activity (nmol/min per mg)	F-actin content (Δ RFI)
None	783 ± 18.2 (100%)	97.1 ± 8.4 (100%)
DNase I	490 ± 9.9 (51%)	54.0 ± 7.6 (56%)
None	955 ± 21.3 (100%)	97.0 ± 7.9 (100%)
Latrunculin A	604 ± 11.4 (63%)	61.5 ± 8.1 (63%)

80 μ M. The result suggests that actin polymerization takes place during the oxidase activation.

Effects of Mg^{2+} and EDTA

F-actin starts to depolymerize when actin concentration is lowered to the critical concentration [17], and critical concentration is drastically decreased by the presence of Mg^{2+} ions [18]. Here we diluted the activated mixture 25-fold with 10 mM Pipes buffer (pH 7) with or without 8 mM $MgCl_2$, or 2 mM EDTA, and examined the time course of the oxidase activity. It should be noted that the control mixture contained 32 μ M $MgCl_2$ when diluted 25-fold (see the Experimental section). Dilution made the deactivation curve biphasic (Figure 4)

Figure 4 Time course of NADPH oxidase in dilution with Mg^{2+} or EDTA

After the cell-free activation the mixture was diluted 25-fold with 10 mM Pipes (pH 7.0) in the absence (low Mg^{2+}) and presence of 8 mM $MgCl_2$ (high Mg^{2+}) or 2 mM EDTA (EDTA) and incubated for 0–60 min at 0 °C. Then the mixture was assayed for O_2^- generation. Data are expressed as means $\pm$ S.D. of three determinations. Initial activities of the diluted mixture with the buffer, the buffer with 8 mM $MgCl_2$, and 2 mM EDTA were 654.7 ± 44.3 , 789.1 ± 58.6 and 558.6 ± 38.7 nmol/min per mg of plasma-membrane protein, respectively.

Figure 5 The effect of free Mg²⁺ concentration on NADPH oxidase stability

(A) NADPH oxidase activity immediately or 30 min after dilution. Cell-free activation was performed in 10 mM Pipes buffer (pH 7.0) containing 0.8 mM MgCl₂, and the mixture was diluted 25-fold with the same buffer containing MgCl₂ or EDTA to afford 6.7 nM–7.99 mM free Mg²⁺ concentration and assayed immediately (○) or after 30 min (△). Data are expressed as means ± S.D. of three determinations. (B) Correlation between free Mg²⁺ concentration and enzyme stability. The stability was estimated from the data in (A) using the following equation: stability (%) = activity (*t* = 30 min)/activity (*t* = 0) × 100.

and the lifetime shorter than that without dilution (Table 1). The *t*_{1/2} values for the first and second phases were 12 and 200 min, respectively. When MgCl₂ was added in the dilution the half-lives for the first and second phases increased to 26 and 320 min. On the other hand, EDTA markedly accelerated the deactivation and the half-lives for the first and second phases decreased to 4 and 60 min, respectively. These results indicate clearly that Mg²⁺ has an ability to prolong the lifetime of the oxidase,

Table 3 Prevention by cross-linking against EDTA-induced NADPH oxidase deactivation and F-actin depolymerization

The cell-free activation mixture was stirred with 20 mM EDC at 0 °C for 30 min. The control mixture (Native) or EDC-treated mixture (EDC) was diluted 25-fold with 20 mM KPB (pH 7) containing 8 mM MgCl₂ or 2 mM EDTA and incubated at 0 °C for 30 min. After the incubation, the mixture was assayed for O₂⁻ generation and subjected to F-actin estimation. Data are expressed as means ± S.D. of three determinations. The values in parentheses are the data as percentages relative to the control activity (960 ± 44.5 nmol/min per mg, set at 100%) or F-actin content of the non-cross-linked, non-diluted, sample (relative fluorescence intensity, ΔRFI, 89.6 ± 5.3, set at 100%).

Sample	Additives in dilution	O ₂ -generating activity (nmol/min per mg)	F-actin content (ΔRFI)
Native	EDTA	243 ± 23.3 (25%)	27.0 ± 5.4 (30%)
	MgCl ₂	758 ± 29.6 (79%)	68.9 ± 6.5 (77%)
EDC	EDTA	615 ± 40.2 (64%)	103 ± 6.6 (115%)
	MgCl ₂	834 ± 29.5 (87%)	92.2 ± 3.5 (103%)

although some populations of the oxidase are relatively stable under the conditions for actin depolymerization.

Effect of free Mg²⁺ concentration

Next we examined the correlation between free Mg²⁺ concentration and oxidase stability. Figure 5(A) shows the oxidase activities at zero time (*t* = 0) and 30 min after dilution at various concentrations of free Mg²⁺. The activity at *t* = 0 increased as free Mg²⁺ concentration was increased. The activity with 7.99 mM Mg²⁺ was about 1.7-fold that with 6.7 nM Mg²⁺. When the stability was defined as activity (*t* = 30)/activity (*t* = 0) × 100 and plotted against free Mg²⁺ concentration, the stability correlated well with free Mg²⁺ concentration (Figure 5B). On the other hand, when Ca²⁺ was used instead of Mg²⁺ the stabilizing effect was hardly observed (results not shown). These results suggest that the effect of Mg²⁺ on the stability of the oxidase is related to the stabilization of actin filaments.

Effect of 1-ethyl-3-(3-dimethylaminopropyl)carbodi-imide (EDC) on deactivation and F-actin loss by dilution with EDTA

To confirm the concept mentioned above, we estimated F-actin content in the diluted mixture in the presence and absence of Mg²⁺. Dilution itself lowered F-actin level to 61% and it was decreased to 30% by addition of EDTA, where the oxidase activity fell to 25% (Table 3). Our earlier study showed that the oxidase activity was remarkably stabilized by cross-linking with EDC. Therefore we examined the effect of EDC on EDTA-induced deactivation and F-actin depolymerization. When the activated mixture was cross-linked with EDC before dilution, F-actin content was not decreased by dilution with EDTA and the activity was largely preserved. The *t*_{1/2} values were much extended in both the presence and absence of Mg²⁺ (Table 1). These results further support the relationship between oxidase stability and F-actin content.

Effect of GTP[S]

Mg²⁺ is known to influence the activation of small GTPases including Rac, Rho and rap1A. Rac is an essential component and rap1A is a possible regulator of the oxidase that is associated with cytochrome *b*₅₅₈. Therefore, we examined whether the effect of Mg²⁺ on stabilization is related to these small GTPases.

GTP[S], a well-established activator for small GTPases (a non-hydrolysable analogue of GTP), was added to the dilution and the stability of the enzyme was examined. The stability was not increased but, in fact, it was somewhat decreased by GTP[S]. The stabilities at 6.6 nM, 0.7 μ M and 7.76 mM free Mg^{2+} were 29, 40 and 56 %, respectively, whereas those without GTP[S] were 38, 56 and 70 %, respectively (Figure 5B).

DISCUSSION

Neutrophils contain high concentrations of actin (approx. 40 μ M in total) [7], and it is actually the most abundant protein in neutrophil cytosol preparation. In the resting cells one-third of total actin is present as F-actin and upon cell activation further polymerization takes place. Actin polymerization has been related to chemotaxis and phagocytosis, both of which are accompanied by motion, but not to the respiratory burst (O_2^- generation).

In the present study, we propose that actin polymerization regulates the lifetime of NADPH oxidase based on the following. (i) DNase I or latrunculin A facilitated the deactivation of NADPH oxidase and actin prevented it. (ii) EDTA accelerated the deactivation in a diluted mixture and Mg^{2+} prevented the deactivation. (iii) The treatment with these agents diminishes the F-actin content. (iv) EDC, a cross-linker, prevents the deactivation and F-actin depolymerization induced by EDTA. It should be stressed that actin polymerization is not essential for the oxidase activation, but is required for the duration of the oxidase.

The biphasic deactivation induced by DNase I, latrunculin or dilution showed that some part of the oxidase activity was resistant to these agents or the condition. This implies the presence of sub-populations of the oxidase complex that do not require or do not depend very much on F-actin for stabilization. It cannot be ruled out, however, that these agents did not depolymerize all the F-actin involved in the oxidase stabilization. Whichever is the case, the contribution of F-actin to the respiratory burst seems important because the net amount of O_2^- generated is governed by the lifetime of the oxidase as well as the catalytic rate.

In the cell-free system, it has been pointed out that there is a requirement for Mg^{2+} at a concentration of several millimolar [19,20]. However, recently it was shown that the optimal Mg^{2+} concentration is much lower (0.1 μ M) when recombinant cytosol phox proteins are used instead of cytosol [21] and, more dramatically, Mg^{2+} is not required when purified cytochrome b_{558} was used instead of the membrane [22]. Thus it seems that Mg^{2+} is not required for the activation in a purified reconstitution system, but is necessary for the maximal activation in a crude system. We speculate that Mg^{2+} is required for F-actin-involved stabilization of the oxidase.

Mg^{2+} ions are thought to stabilize F-actin by shifting the equilibrium from G- to F-actin. The equilibrium between G- and F-actin depends critically on the concentration of bivalent cations such as Mg^{2+} and Ca^{2+} [16]. In the absence of bivalent cations, actin is essentially monomeric. Addition of bivalent cations markedly decreases the critical concentration for actin polymerization. For example, the critical concentration is approx. 0.05 μ M for Mg^{2+} -bound actin and approx. 0.3 μ M for Ca^{2+} -bound actin [18]. In our system, total actin concentration was estimated to be 2 μ M in the activation mixture and 0.08 μ M after dilution, which is higher than the critical concentration with Mg^{2+} and lower than that with Ca^{2+} . This is consistent with the result that Mg^{2+} , and not Ca^{2+} , had a significant effect on the oxidase stability.

As Mg^{2+} facilitates the GDP/GTP exchange reaction on small GTPases at a low concentration (< 0.1 μ M), additional activation of Rac might stabilize the oxidase. However, the Mg^{2+} concentrations used in our system are much higher (> 1 mM) and at such a concentration the nucleotide exchange is not facilitated [23]. Thus it is unlikely that Rac is involved in Mg^{2+} -supported stabilization. Actually, the addition of GTP[S] to the dilution mixture did not increase, but rather decreased, the enzyme stability to some extent.

We used SDS as an activator for cell-free activation of NADPH oxidase. Since the system was established, the anionic amphiphile has often been used for the activation system, as well as arachidonate [3]. Nevertheless, the exact role of SDS in the activation is not clear. It has been suggested that SDS mimics p47^{phox} phosphorylation catalysed by a protein kinase by providing negative charges to the protein. However, a pure system, which includes a large excess of p67^{phox} and Rac over cytochrome b_{558} , does not need p47^{phox} but needs SDS to be activated [24], suggesting that SDS also acts on other phox protein(s).

One of the most interesting findings here is that F-actin increases during NADPH oxidase activation. Although the exact mechanism for the phenomenon is not clear, the conditions for the oxidase activation could be suitable for actin polymerization, i.e. the requirement of Mg^{2+} , GTP, small GTPase such as Rac, and high concentrations of G-actin (cytosol). It was reported that Rho GTPases, including Rac, Rho or Cdc42, induce actin polymerization in neutrophils or other cells [25,26]. Thereby it is plausible that the activated Rac or other GTPase may induce actin polymerization. In this context, it is of interest that SDS is able to activate Rho GTPases by dissociating the complex with Rho GDI (GDP-dissociation inhibitor) [27,28] because in the cytosol Rac GTPase is complexed with Rho GDI [29].

Cytochalasins, which are often used to depolymerize F-actin, had no effect on the oxidase stability (results not shown). In contrast, latrunculin A facilitated the deactivation of the oxidase. It is known that latrunculin, and not cytochalasin, depolymerizes F-actin that lies beneath the plasma membrane, i.e. cortical actin [30,31]. This might be due to the difference in action mechanism between cytochalasin and latrunculin. Cytochalasins bind to the barbed end of F-actin to block filament elongation while latrunculin depolymerizes F-actin by a similar mechanism to DNase I [31]. Thus the difference in the effects of cytochalasins and latrunculin may suggest that the F-actin that stabilizes the oxidase is cortical actin.

How can actin filaments stabilize neutrophil NADPH oxidase? The interaction between cytoskeleton and the oxidase components has been reported, which include p47^{phox}, p67^{phox}, [9,32], Rac [33] and cytochrome b_{558} [9]. Coronin, an actin-binding protein, was also reported to interact with p67^{phox} and p40^{phox} [34]. So it is reasonable to speculate that the cytosol phox proteins link to actin filaments, either directly or indirectly. On the other hand, cortical actin anchors to the plasma membrane through ERM proteins (ezrin, radixin and moesin) and a membrane-spanning protein, CD44 [35]. Therefore, actin filaments may stabilize and immobilize the oxidase complex as a scaffold on the plasma membrane.

In summary, the present work indicates that during the activation of the oxidase actin filaments grow on the plasma membrane and stabilize the oxidase complex. This suggests that actin polymerization and depolymerization regulate the lifetime of the oxidase in neutrophils.

Finally what is the physiological significance of actin regulation of NADPH oxidase? Active oxygen species, such as O_2^- and its derivatives, are microbicidal but also toxic to the host cells. Complex formation on the membrane cytoskeleton may help to

restrict the area of the respiratory burst and avoid unfavourable 'firing'. On the physiological relevance of actin in the respiratory burst, it is of interest that Nunoi et al. reported recently of a patient with a mutated β -actin gene showing impaired O_2^- generation in neutrophils [36].

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