Spermine suppresses the activation of human neutrophil NADPH oxidase in cell-free and semi-recombinant systems

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Spermine, a cellular polyamine, down-regulates O_2^- generation in human neutrophils stimulated by receptor-linked agonist [Ogata, Tamura and Takeshita (1992) Biochem. Biophys. Res. Commun. **182**, 20–26]. In this study, to elucidate the mechanism for the inhibition, the effect of spermine on cell-free activation of the O_2^- generating enzyme (NADPH oxidase) was examined. Spermine suppressed the SDS-induced activation of NADPH oxidase in a dose-dependent manner with an IC_{50} of 18 μM. The inhibition was specific for spermine over its precursor amines, spermidine and putrescine. Spermine did not alter the K_m for NADPH or the optimal concentration of SDS for activation. The amine was inhibitory only when added before activation, indicating that it affects the activation process rather than the enzyme's activity. An increased concentration of cytosol partly prevented the inhibition by spermine. In semi-recombinant cell-free system, spermine inhibited the activation of NADPH oxidase as effectively as in the cell-free system ($IC_{50} = 13$ μM). Pre-treatment of each recombinant cytosolic component with spermine revealed that they (especially p67phox) are sensitive to spermine. These results suggest that spermine interacts with cytosolic component(s) and impairs the assembly of NADPH oxidase.

INTRODUCTION

Neutrophils play an important role in host defence against microbial infection. On phagocytosis of microbes such as bacteria or fungi, neutrophils produce a large amount of superoxide (O_2^-) ions with the secondary generation of more toxic species (i.e. H_2O_2, HOCl, and $^{\bullet}OH$) to attack the phagocytosed pathogens [1,2]. The enzyme that responds for O_2^- generation is called NADPH oxidase (or respiratory burst oxidase), which converts O_2 to O_2^- by using electrons from NADPH. The enzyme is dormant until the cells undergo phagocytosis or are activated by a chemoattractant such as fMet-Leu-Phe or C5a [3,4].

NADPH oxidase can be activated in a cell-free system consisting of plasma membrane and cytosol in the presence of anionic amphiphile [3]. The enzyme components are separately located in the membrane and cytosol, and become associated on activation. The complementation study with fractions from CGD (chronic granulomatous disease) patients and the reconstitution with pure recombinant proteins have revealed that membrane-bound cytochrome b_{558} (cyt.b_{558}) and three cytosolic factors ($p47_{phox}$, $p67_{phox}$, p21rac) are essentially involved [5]. However, the mechanism for the activation has not been fully elucidated.

In contrast to the activating ability of lipophilic anions such as SDS, arachidonate [3] or phosphatidic acid [6], lipophilic cations have been shown to inhibit NADPH oxidase activation. These include monoalkylamines [7–9] and azelastin [10], an antiallergic drug, but they are xenobiotic molecules and the physiological relevence of the phenomenon is unknown. Lambeth's group reported the inhibitory effect of sphinganine, a biogenic amine, on NADPH oxidase activation in whole cells [11] and ascribed it to the inhibition of protein kinase C. The inhibition of NADPH oxidase by physiological amines is of interest in relation to the regulation of O_2^- generating activity.

Spermine is a biogenic polyamine abundantly present in mammalian cells. The cellular concentration of this aliphatic amine is reportedly in the range of 0.1–2 mM in normal human tissues (such as liver, kidney and brain) [12,13]. The amine is biosynthesized from arginine via ornithine, putrescine and spermidine. It is known that spermine promotes cell growth and maturation. The amine is thought to facilitate protein synthesis by interacting with nucleic acids [12]. In addition, spermine has been reported to exert either stimulatory or inhibitory effects on specific enzymes. For example, whereas spermine can stimulate a tyrosine kinase from porcine spleen [14] as well as the GTPase activity of a heterotrimeric G-protein from calf brain [15], it can also inhibit superoxide dismutase in hamster spermatozoa [16], phosphoinositide-phospholipase C in a tumour cell line [17], and phosphatidate phosphohydrolase from rat liver [18]. It is interesting that most of these enzymes are thought to be involved in signal transduction.

Previously we reported that spermine suppresses O_2^- generation by neutrophils induced by a receptor-linked agonist such as fMLP or concanavalin A, whereas that induced by phorbol ester, diacylglycerol, or arachidonate is not affected [19]. On the basis of the results, we suggested that spermine inhibits the activation process rather than the enzyme itself. In the present study, to elucidate the mechanism of spermine inhibition, we examined the effect of spermine on cell-free activation and semi-

Abbreviations used: cell-free system, cell-free system containing cytosol; cyt.b_{558}, cytochrome b_{558}; GTP-γS, guanosine 5′-*O*-(3-thiotriphosphate); semi-recombinant (cell-free) system, cell-free system containing recombinant cytosolic components.

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recombinant activation of NADPH oxidase, and found that the amine interacts with the oxidase components to impair the activation of the oxidase.

MATERIALS AND METHODS

Materials

Spermine tetrahydrochloride, spermidine trihydrochloride, cytochrome *c* (horse heart, type VI), trypsin (bovine pancreas, *N*-tosyl-L-phenylalanine chloromethylketone-treated, type XIII), trypsin inhibitor (soybean, type I-S), and superoxide dismutase (bovine erythrocyte) were obtained from Sigma (St. Louis, MO, U.S.A.). Putrescine dihydrochloride was purchased from Wako Pure Chemicals (Osaka, Japan). NADPH was purchased from Oriental Yeast Co. Ltd. (Tokyo, Japan). All other reagents were the best grade commercially available.

Isolation and subcellular fractionation of human neutrophils

Blood (total 450 ml) was drawn from two healthy volunteers, and $(0.8–1.2) \times 10^9$ neutrophils were separated as described previously [20]. The separated cells were suspended in 10 ml of relaxation buffer (100 mM KCl, 3.5 mM $MgCl_2$, 1 mM ATP, 10 mM potassium phosphate buffer, pH 7.3), disrupted by cavitation under nitrogen pressure, and fractionated as described previously [21]. The cytosol fraction was concentrated 15–20-fold by using an Amicon concentrator with a PM-10 membrane. The plasma membrane fraction was centrifuged at 140000 ***g*** for 1 h and the pellet resuspended in 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 0.34 M sucrose, 5 mM potassium phosphate buffer, pH 7.0. The cytosol and the plasma membrane fractions were stored at −80 °C until use.

Cell-free activation of NADPH oxidase and assay for O_2^- generation

Plasma membrane (18 μg of protein, 6×10^6 cell equivalents) and cytosol (165 μg of protein, 6×10^6 cell equivalents) were mixed in 1 ml of ice-cold assay buffer (8 mM $MgCl_2$, 20 mM potassium phosphate buffer, pH 7.0) and incubated at 0 °C for 1 min with or without spermine. To the mixture SDS was added at 100 μM and the mixture was kept at 0 °C for 10 min. Superoxide generation was assayed as superoxide dismutase-inhibitable cytochrome *c* reduction at 25 °C, with a Shimadzu spectrophotometer MPS-2000 or 160A as described previously [21]. Aliquots (0.5 ml) of the activated cell-free mixture were transferred into two cuvettes, both containing 80 μM cytochrome *c*, with the reference cuvette containing superoxide dismutase (100 μg/ml). The cuvettes were then warmed to 25 °C for 1 min, and transferred to the thermostated cuvette holder of the spectrophotometer where cytochrome *c* reduction was monitored after the addition of 200 μM NADPH to both cuvettes. O_2^- generating activity was expressed as nmol of O_2^- formed per min per mg of plasma membrane protein.

Expression and purification of recombinant proteins, p47phox, p67phox and p21rac

Recombinant p47phox and p67phox were expressed in Sf9 cells by using recombinant vaculovirus and purified as described previously [22]. As for p21rac, we used a mutant form of rac 1, rac 1(C189S) in which Cys-189 is replaced by serine. The mutant was shown to be fully active and more stable than wild type [23]. rac 1(C189S) was expressed in *Escherichia coli* BL21 by using pGEX-2T vector and purified as described previously [23] except that the final preparation did not contain dithiothreitol.

Semi-recombinant cell-free activation

Semi-recombinant cell-free activation was performed as described [24] with several modifications. Recombinant p47phox (0.9 μg), p67phox (0.9 μg), and rac 1(C189S) (1.2 μg) preloaded with 0.1 mM guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γS), were mixed with plasma membrane (6.5 μg) in 1 ml of 20 mM potassium phosphate buffer, pH 7.0, containing 8 mM $MgCl_2$, 10 μM GTP-γS and 0–160 μM spermine. The mixture was incubated at 0 °C for 3 min and then supplemented with 100 μM SDS and incubated at 0 °C for a further 10 min. The mixture was warmed to 25 °C for 1 min and assayed for O_2^- generation as described above (cell-free activation). For individual treatment of recombinant factors with spermine, each recombinant cytosolic factor (0.9 μg) was treated with spermine (0–1.66 mM) for 5 min at 0 °C and an aliquot (5 μl) of the mixture was used for semi-recombinant cell-free activation described above.

Spermine content in neutrophils and the cytosol fraction

The extraction of spermine and HPLC analysis were performed as described previously [25]. Neutrophil homogenate or cytosol fraction was extracted with 0.2 M trichloroacetic acid and centrifuged at 27000 ***g*** for 15 min at 4 °C. The supernatants were analysed on a Toyo Soda HPLC system on TSK gel IEX215 (4 mm × 80 mm) at 50 °C. After reaction with *o*-phthalaldehyde, the amines in the effluent were detected by a fluorescence detector.

RESULTS

Inhibition by spermine of SDS-induced activation of NADPH oxidase

Figure 1 shows the effect of spermine on the activation of NADPH oxidase. When plasma membrane and cytosol were

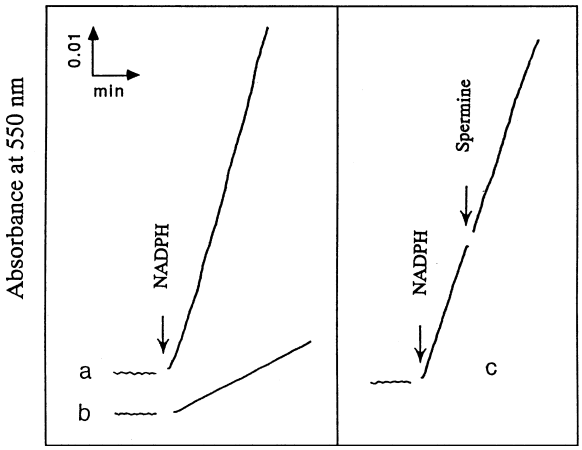

Figure 1 Effect of spermine on cell-free activation of NADPH oxidase by SDS

Plasma membrane (18 μg) and cytosol (165 μg) were preincubated without (trace **a**) or with (trace **b**) spermine (40 μM) in 1 ml assay buffer for 1 min at 0 °C, and then activated with 100 μM SDS for 10 min at 0 °C. The superoxide generating activity was assayed as described in the Materials and methods section. In trace **c**, spermine was added 1 min after the addition of NADPH to the reaction mixture. This is a typical result from three experiments.

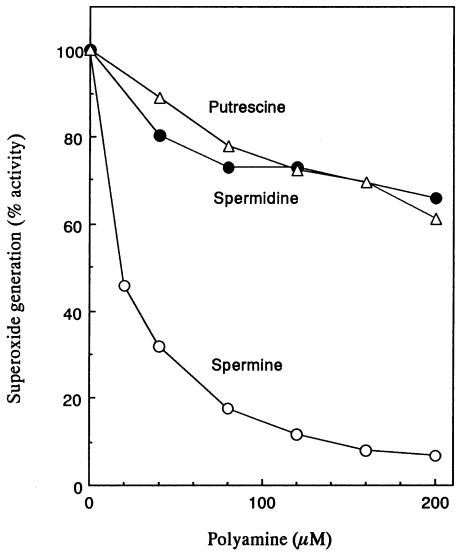

Figure 2 Effect of several polyamines on cell-free activation of NADPH oxidase

Plasma membrane and cytosol were preincubated with spermine, spermidine or putrescine at 0–200 μM for 1 min at 0 °C. The activation with SDS and the assay for superoxide generation were performed as described for Figure 1. The control activity was 112 $\pm$ 5.7 nmol per min per mg of membrane protein. Symbols represent the averages for three experiments.

mixed with SDS in the absence of spermine, the observed O_2^- generation was 162 nmol per min per mg of plasma membrane protein (trace a), whereas when a mixture of plasma membrane and cytosol was treated with 40 μM spermine before activation, O_2^- generating activity decreased to 32 % of the control (trace b). In contrast, spermine did not influence the activity when added after activation (trace c). These results indicate that spermine affects the activation process rather than the activity itself.

Concentration dependence of the inhibition of NADPH oxidase by spermine and other polyamines

Figure 2 shows the concentration dependence of the inhibition of SDS-induced activation by spermine. Spermine inhibition was dose-dependent and the IC_{50} value of the inhibition was estimated as 18 μM. To examine whether the inhibition is specific for spermine, the effect of other polyamines, spermidine and putrescine (precursors of spermine) were also examined (Figure 2). The inhibitions by these amines were modest and much weaker than that by spermine. Even at 200 μM, at which concentration spermine almost completely blocks the activation, spermidine and putrescine inhibit the activation by only 33 and 39 %, respectively. This indicates that the inhibition of spermine is specific over its precursor amines.

Effect of spermine on the kinetic parameters of NADPH oxidase

To examine whether spermine impairs the ability of NADPH binding of the enzyme, K_m values for NADPH of the oxidase activated in the presence and absence of spermine were determined. As shown in Table 1, the K_m for NADPH was not altered by spermine treatment before the activation, although $V_{max.}$ was considerably decreased. This indicates that spermine does not change NADPH-binding site of the oxidase.

Table 1 Kinetic parameters of NADPH oxidase activated in a cell-free system in the absence and presence of spermine

Cell-free activation was performed in the absence or presence of spermine (40 μM), and assays were done with various NADPH concentrations from 10 to 400 μM. Other assay conditions are as described in the Materials and methods section. K_m and $V_{max.}$ values were determined by using a non-linear least-squares fit of the data.

Condition	K_m for NADPH (μM)	$V_{max.}$ (nmol O_2^- per min per mg)
Control	31.8 $\pm$ 4.6	194.5 $\pm$ 12.0
Spermine	27.2 $\pm$ 1.2	88.5 $\pm$ 1.3

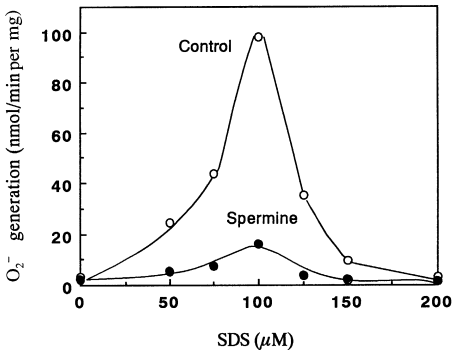

Figure 3 Effect of spermine on concentration dependence on SDS of the activation of NADPH oxidase

The membrane and cytosol were pretreated with spermine (40 μM) and activated with various concentrations of SDS. The assay for O_2^- generation was performed as described in the Materials and methods section. Symbols represent the averages for three experiments.

Effect of spermine on the optimal concentration of SDS for activation

As spermine is a cationic molecule and the activator SDS is an anion, one might think that the inhibition is due to the neutralization of SDS by spermine. To clarify this point we examined the concentration dependence on SDS of the activation in the presence or absence of spermine (Figure 3). The optimal concentration of SDS for activation was not altered by the presence of spermine. This result excludes the possibility that spermine interacts directly with SDS to reduce the effective concentration of the activator.

Effect of cytosol concentration on the inhibition of SDS-induced activation

Table 2 shows the effect of cytosol concentration on the inhibition by 40 μM spermine. When the cytosol concentration was increased from 0.5 to 4 cell equivalents at constant membrane amount (6×10^6 cell equivalents), the control activity increased from 45 to 410 nmol per min per mg of plasma membrane protein. The inhibition by spermine decreased from 73 % to 45 %. We examined the protecting effect of several small molecules on the inhibition, which might be included in the cytosol fraction, such as FAD, ATP and GTP (actually GTP-γS was examined), and found no protecting effect of these molecules (results not shown). Therefore we next sought to determine whether the protecting effect observed might be mediated by a protein factor. When added to the cell-free system, trypsin-

Table 2 Effect of cytosol concentration on the inhibition by spermine

Cell-free activation was performed in the absence or presence of spermine (40 μM) with different amounts of cytosol expressed as the ratio of cell equivalents at constant membrane amount (6×10^6 cell equivalents). Samples were assayed for superoxide generation as described in the Materials and methods section. O_2^- generating activities are expressed as nmol O_2^- per min per mg of membrane protein. The activities are shown as means $\pm$ S.E.M. for three determinations.

Cytosol (ratio)	O_2^- generating activity		Activity, spermine/control (%)
	Control	Spermine	
0.5	38.3 $\pm$ 5.8	10.2 $\pm$ 3.4	26.6
1.0	172.0 $\pm$ 2.7	49.1 $\pm$ 2.4	28.5
2.0	312.5 $\pm$ 0.4	116.5 $\pm$ 6.1	37.3
3.0	354.5 $\pm$ 9.9	164.9 $\pm$ 7.8	46.5
4.0	417.7 $\pm$ 1.3	230.5 $\pm$ 19.3	55.2

Table 3 Effect of trypsin treatment on the protection by cytosol against spermine inhibition

The cell-free mixture contained 6×10^6 cell equivalents of the membrane fraction and the indicated amount of cytosol (native or treated). Cytosol was treated with 1 mg/ml trypsin for 10 min at 37 °C and then supplemented with trypsin inhibitor at 0.5 mg/ml and used in the assay [trypsin (+)]. As a control, cytosol was treated in the same way, except that trypsin was omitted [trypsin (−)]. O_2^- generating activities are expressed as nmol O_2^- per min per mg of membrane protein. The activities are means $\pm$ S.E.M. for three determinations.

Cytosol (eq.)			O_2^- generating activities		Activity, spermine/control (%)
Native	Treated	Total	Control	Spermine	
1	−−	1	161.9 $\pm$ 2.8	52.9 $\pm$ 3.1	32.7
4	−−	4	493.4 $\pm$ 8.7	272.6 $\pm$ 4.7	55.2
1	Trypsin (+)	4	148.7 $\pm$ 0.3	46.8 $\pm$ 4.9	31.5
1	Trypsin (−)	4	169.7 $\pm$ 1.6	93.0 $\pm$ 2.6	54.8

treated cytosol showed neither additional activity nor protecting effect against spermine (Table 3). This indicates that trypsin eliminated the ability of cytosol to prevent the inhibition, and that the preventing factor(s) is a protein. In contrast to cytosol concentration, increased membrane concentration did not show any protecting effect on the inhibition (results not shown).

Effect of spermine on semi-recombinant cell-free activation of NADPH oxidase

The results described above show that the site(s) of spermine action is/are located at cytosolic protein(s). Therefore, to establish whether the protein is oxidase cytosolic components or another regulatory protein (see the Discussion section), we examined the effect of spermine on semi-recombinant cell-free activation [24] of the oxidase. As shown in Figure 4, spermine strongly inhibited the activation of the oxidase in the semi-recombinant system. The inhibition was dose-dependent with an IC_{50} of 13 μM, which is similar to that (18 μM) for cell-free activation with cytosol, although above 60 μM the activity was not greatly influenced, and nearly 25 % activity remained. These results suggest that spermine inhibition is largely due to its interaction with the cytosolic component(s) of the oxidase.

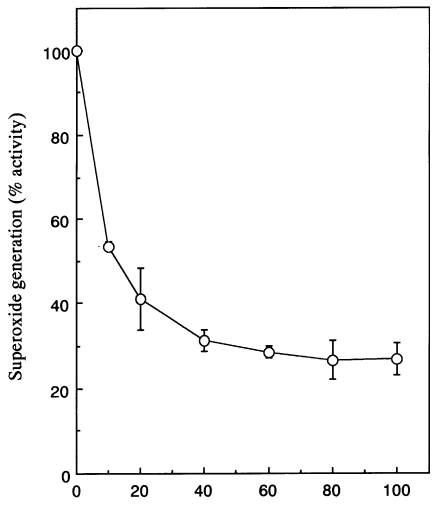

Figure 4 Inhibition of semi-recombinant cell-free activation of NADPH oxidase by spermine

Plasma membrane and three recombinant cytosolic components [$p47_{phox}$, $p67_{phox}$, and rac 1(C189S)] were mixed and activated by SDS in the presence or absence of 40 μM spermine. Other experimental conditions are as described in the Materials and methods section. Symbols and error bars represent means $\pm$ S.E.M. for three determinations. The control value was 90.7 $\pm$ 7.2 nmol per min per mg of membrane protein.

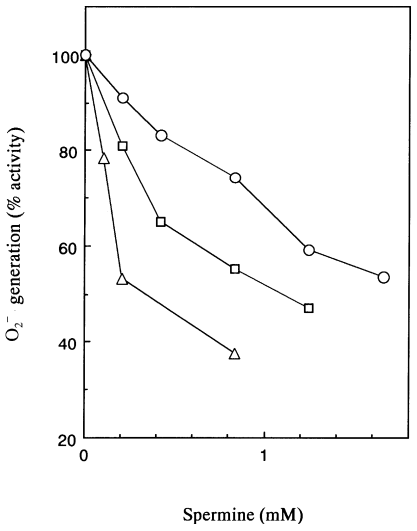

Figure 5 Effect of pretreatment of individual recombinant cytosolic components with spermine

Each recombinant component, p47phox (□), p67phox (△), or rac 1(C189S) (○), was pretreated with spermine (0–1.66 mM) and used in the semi-recombinant cell-free activation system. Other experimental conditions are as described in the Materials and methods. Each symbol shows the average from two or three experiments. The control value was 94.8 $\pm$ 4.5 nmol per min per mg of membrane protein.

Effect of separate spermine treatment of recombinant cytosolic factors

To clarify which cytosolic factor(s) is/are the target of spermine, each recombinant protein was treated with spermine and then

Table 4 Inhibition by spermine of rac activation by GTP-γS

Recombinant rac 1(C189S) (1.2 μg) was treated with spermine (0–0.83 mM) for 5 min at 0 °C before or after preloading of GTP-γS and used in the semi-recombinant activation system. Other assay conditions are as described in the Materials and methods section. The control activity was 100.6 ± 13.8 nmol per min per mg of membrane protein. Values are means ± S.E.M. for three determinations. Results shown are representative of two independent experiments.

	O_2^- generating activity (% of control)		
Spermine concn. (mM) ...	0.2	0.42	0.83
Before GTP-γS	69.9 ± 3.5	54.3 ± 7.2	41.8 ± 4.0
After GTP-γS	93.0 ± 5.1	83.2 ± 6.0	74.3 ± 16.1

used in the semi-recombinant cell-free system. As shown in Figure 5, p67phox was the most sensitive to spermine and p47phox the second. When p67phox, p47phox or rac 1(C189S) was pretreated with 0.2 mM (or 0.83 mM) spermine, the activity was 53 %, 81 % or 91 % (38 %, 55 % or 74 %) respectively. In these experiments, the concentration of spermine was greatly diluted in the activation mixture (e.g. 0.83 mM spermine in pretreatment corresponds to 4 μM in the final incubation), and under this condition, the activity was not much influenced (Figure 4). When plasma membrane was pretreated with spermine, no prominent inhibition was observed. The treatment of the membrane with 0.42 or 0.83 mM spermine resulted in 94.5 % or 83.8 % activity respectively. This indicates that spermine acts on the cytosolic component(s) of NADPH oxidase (especially p67phox).

Effect of spermine on the activation of rac 1(C189S)

In semi-recombinant cell-free activation, rac 1(C189S) is required to be preloaded with GTP-γS [23]. Here we examined whether spermine interferes with the activation of the G-protein by GTP-γS: rac 1(C189S) was treated with spermine before activation with GTP-γS. As shown in Table 4, the oxidase activation was considerably impaired by this treatment (IC_{50} 0.6 mM). This result is quite different from the case in which the rac was treated after GTP-γS preloading (Figure 5), and suggests that spermine interferes with the binding of GTP-γS to the G-protein.

Estimation of spermine content in human neutrophils

To consider the biological relevance of spermine inhibition, we determined spermine content in neutrophils. It was found that human neutrophils contain 2.3 nmol spermine per 10^7 cells. This means that total cellular concentration in neutrophils is 0.46 mM (taking an average volume of neutrophils as 0.5 ml per 10^9 cells). In addition, spermine content of the cytosol fraction was determined to be 0.2 nmol spermine per 10^7 cells. This would correspond to 40 μM if cytosol volume were equal to the total cell volume. However, the former is apparently much smaller than the latter (for example, the cytosol volume is estimated to be 54 % of total cell volume in typical liver cells [26]), so cytosol should contain much higher concentration of spermine than 40 μM.

DISCUSSION

In this paper we demonstrated first the suppression of the activation of NADPH oxidase in a cell-free system by spermine. The kinetic studies showed that the inhibition was not caused by neutralization of SDS or by altering the K_m value for NADPH. Also the inhibition did not occur when spermine was added after activation. These results shows that spermine acts on the activation process rather than the oxidase activity.

In attempts to clarify the mechanism of inhibition by spermine, we found that the inhibition was restored by increasing cytosol concentration. This result and the abolition by trypsin of the protecting ability of cytosol suggest that the protecting factor is a cytosol protein and probably that the protein is the site for spermine inhibition. As the cytosol fraction contains many kinds of cytosol protein, it is difficult to identify the factor. Therefore, to simplify the system and establish whether spermine inhibition occurs at the cytosolic components of NADPH oxidase or other possible regulatory protein (such as GDP dissociation inhibitor, a certain protein kinase or cytoskeleton) [27], we next examined the effect of spermine on a semi-recombinant cell-free system containing recombinant p47phox, p67phox, and rac 1(C189S) [24].

Spermine also inhibited the oxidase activation in the semi-recombinant cell-free system, with an IC_{50} of 13 μM, similar to that for cell-free system. This indicates that the site of action by spermine is a cytosolic component(s) of the oxidase although we could not exclude the possibility that spermine has an additional effect on other cytosolic protein(s) because the inhibition in the semi-recombinant system was relatively modest at higher concentrations of spermine. Pretreatment of each recombinant protein with spermine shows that p67phox is the most sensitive to the amine, p47phox is the second, and rac is not much influenced by pretreatment at 1 mM. This indicate that the inhibition by spermine is mainly caused by its binding to p67phox and also p47phox. In this context it should be noted that p67phox is an acidic protein with a large anion cluster at its C-terminal region [28], and p47phox also has an anion cluster in the middle although the whole protein is basic [29]. Interestingly, both of these anion clusters are located in SH3 regions, which are thought to interact with each other or with a cyt.b_{558} subunit [30,31]. The binding of spermine to these sites may impair the interaction between the oxidase components.

Although rac 1(C189S) is much less sensitive to spermine than other cytosolic factors, when treated with the amine before GTP-γS preloading, the oxidase activation was significantly inhibited. This suggests that spermine interferes with the binding of GTP-γS to rac 1(C189S) (in fact GDP–GTP-γS exchange) to impair the activation of the G-protein. It is plausible that spermine binds to GTP-γS and reduces the concentration of free nucleotide. Another, more interesting, explanation is that spermine binds to the rac–GDP complex and interferes with the exchange of GDP with GTP-γS. Further study will be required to clarify the mechanism of the inhibition of rac activation. Although the inhibition of rac activation by polyamine is quite interesting, it does not account for the inhibition of the semi-recombinant cell-free system described here, in which spermine was added after rac activation. Instead, the inhibition may explain the inhibition by spermine of GTP-γS activation of the oxidase in permeabilized cells (19).

Recently several cationic peptides have been shown to inhibit NADPH oxidase activation. Tal et al. [32] reported that defensin, a cellular cationic peptide, inhibits the activation of the oxidase, and the inhibition was relieved by increasing the concentration of p47phox. Also the synthetic peptides RGVHFIF (which corresponds to the C-terminus of the cyt.b_{558} β-subunit [33]) and CPPPVKKRKRK (the C-terminus of rac 1 [23]) strongly inhibit the oxidase activation. The former peptide was postulated to bind to p47phox [33], and the latter peptide to cyt.b_{558} [23] or possibly some other membrane constituent [34]. Although the

mechanism for the inhibition by these peptides is not completely elucidated, all these compounds have properties in common with spermine, i.e. they are polycationic and aliphatic. Thus the inhibition by some of these peptides may be related in mechanism to that by spermine, although the binding to a membrane component is unlikely for spermine inhibition (see results).

What is the physiological relevance of spermine inhibition? Based on the IC_{50} for the inhibition and the estimated spermine content in the cell, it is reasonable to postulate that spermine regulates NADPH oxidase activation in intact neutrophils. This concept may be supported by the following. (1) Only spermine, and not precursor amines, is effective on the inhibition, which is the terminal product in polyamine metabolism [12]. (2) The intracellular spermine level is strictly controlled through the activities of ornithine decarboxylase and polyamine-interconverting enzymes [12]. (3) The level of spermine can vary with cell conditions [13] or hormone actions [35]. In addition, it is suggested that the free spermine level might change depending on the concentration of nucleotides present (e.g. ATP) [36]. The physiological role of spermine in the regulation of NADPH oxidase seems an interesting problem to be clarified.

In summary, we have found that spermine inhibits the activation of NADPH oxidase in a cell-free system and a semi-recombinant system. The results show that spermine acts on p67phox and probably on p47phox to impair the assembly of NADPH oxidase components.

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