Biochemical and Biophysical Research Communications **257,** 124–128 (1999)
Article ID bbrc.1999.0428, available online at http://www.idealibrary.com on IDEAL®

Uncompetitive Inhibition of Superoxide Generation by a Synthetic Peptide Corresponding to a Predicted NADPH Binding Site in gp91-*phox*, a Component of the Phagocyte Respiratory Oxidase

Tomoko Tsuchiya, Shinobu Imajoh-Ohmi, Hiroyuki Nunoi,[1] and Shiro Kanegasaki
Institute of Medical Science, University of Tokyo, Tokyo 108-8639, Japan

Received February 17, 1999

The large subunit of cytochrome b558, gp91-*phox,* is believed to play a key role in superoxide generation in neutrophils by accepting electrons from NADPH and donating them to molecular oxygen. We found that a peptide corresponding to a predicted NADPH binding site in gp91-*phox* inhibited superoxide generation in a cell-free system consisting of neutrophil membrane and cytosol. Minimum essential sequence for the inhibition was KSVWYK, which corresponded to residues 420-425 (IC50 = 30 μM). Unlike other peptides known to inhibit the reaction, this peptide was effective even when added to the system after activation or to activated membrane from stimulated neutrophils. Furthermore, the peptide inhibited superoxide generation in a membrane system activated without cytosol. Kinetic analysis revealed that the peptide inhibited the reaction *uncompetitively.* These results suggest that the peptide combines with the activated cytochrome b558–NADPH complex and thereby inhibits electron transfer from NADPH to molecular oxygen.
© 1999 Academic Press

Phagocytes and B lymphocytes have an enzyme system that catalyzes one-electron reduction of molecular oxygen at the expense of cellular NADPH as an electron donor (1-4). The system is activated during phagocytosis or upon exposure to certain soluble stimuli. Molecular oxygen rapidly consumed by the cells is stoichiometrically converted to superoxide anion, which is released to the outside of the cells or inside of the phagosome (5). The anion is in turn converted to hydrogen peroxide and other compounds that may be used for killing microorganisms in phagocytes.

This process is designated as the "respiratory burst" (6). The importance of the respiratory burst in host defense is exemplified by the disorder chronic granulomatous disease. Patients with this disease suffer from recurrent, life-threatening infections because phagocytes cannot kill microorganisms due to a defect in the respiratory burst (7, 8).

The superoxide generating system consists of at least five components; a cytochrome b558 composed of 91- and 22-kDa subunits (termed gp91-*phox* and p22-*phox,* respectively) that resides in the membrane, 47- and 67-kDa cytoplasmic proteins (designated as p47- and p67-*phox*, respectively) and the low molecular weight GTP-binding protein, Rac (1). When activated, the cytosolic proteins migrate to the membrane and form active enzyme complex, where the cytochrome plays structurally central role for the assembly (9, 10).

Cytochrome b558 has a very low redox potential (11) and is believed to be a terminal oxidase. Based on sequence homology between gp91-*phox* and the NADPH and FAD binding regions of several flavoproteins (12-14), and on the results of labeling experiments of the purified cytochrome with an NADPH analogue and FAD (15), the cytochrome is predicted to be a flavocytochrome. The cytochrome, therefore, seems to have all necessary prosthetic groups to accept electrons from NADPH in the cytoplasm and to donate them to molecular oxygen at the extracellular site. In fact, it was shown that delipidated and then relipidated cytochrome b558 isolated from membrane possessed NADPH dependent superoxide generating ability (16, 17), though activity is lower than that of the full system. The cytosolic components are thought to activate the cytochrome by inducing conformational changes through direct binding interactions. Using various synthetic peptides, the regions in gp91-*phox* that interact with the cytoplasmic proteins (18-25) and its topology in membrane have been proposed previously (26).

In this paper, we focus our attention on the predicted NADPH binding sites in gp91-*phox* and study whether synthetic peptides corresponding to these regions af-

[1] To whom correspondence should be addressed at Department of Pediatrics, Kumamoto University Medical School, Kumamoto, Japan.

0006-291X/99 $30.00
Copyright © 1999 by Academic Press
All rights of reproduction in any form reserved.

TABLE 1

Effect of Various Synthetic Peptides Corresponding to the Predicted NADPH Binding Regions of gp91-*phox* on Superoxide Generation in a Cell-Free System

Name	Residues	Sequence	IC50 (μM)
L410	410-415	GIGVTP	>250
L418	418-435	ILKSVWYKYCNNATNLKL	12 ± 3.2
L441	441-450	FYWLCRDTHA	58 ± 7.6
L504	504-512	GLKQKTLYGR	>250
L531	531-546	RIGVFLCGPALAETL	>250
L559	559-565	RGVHFIF*	45 ± 3.1
		RGVHAIF#	>250

*,# The peptide corresponding to residues 559-565 of gp91-*phox* and the peptide with the same sequence except that phenylalanine residue was substituted with alanine were used as positive and negative controls for inhibition (18, 22).

fected superoxide generation in a cell free system from human neutrophils.

MATERIALS AND METHODS

Materials. Peptides were synthesized by a solid-phase method on a Shimazu PMMS-8 synthesizer, deprotected and purified as described previously (19). n-Heptyl-β-D-thioglucoside was obtained from Dojindo Laboratories and Cypridina Luciferin analog, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-α]pyrazin-3-one (CLA) was from Tokyo Kasei Kagaku. Phosphatidylcholine (PC: type-μ from soybean), phosphatidic acid (PA: from egg yolk), superoxide dismutase (SOD), phorbol-12-myristate-13-acetate (PMA), NADPH, GTPγS and ferricytochrome c were from Sigma.

Isolation of neutrophils and preparation of cell-free system of oxidase axtivation. Human neutrophils were obtained from buffy coat (19), and membrane and cytosol were prepared as described previously (20). The relaxation buffer used contained 100 mM KCl. 3 mM NaCl, 3.5 mM $MgCl_2$, 10 mM PIPES and 125 μM EGTA. Membrane was solubilized with 1% n-heptyl-β-D-thioglucoside according to the method described by Miki et al. (27). Concentrations of the solubilized membrane and the cytosol, respectively, were adjusted to 10^8 cell equivalent/ml and 10^9 cell equivalent/ml, and these fractions were stored at −80°C until use. All procedures were carried out at 4°C. The activated membrane (28) was obtained from neutrophils pretreated with 100 ng/ml PMA at 37°C for 10 min. This membrane fraction contains the activated superoxide generating system consisting of cytochrome b558 and associated cytoplasmic proteins.

Preparation of membrane activated without cytosolic proteins. Membrane that can be activated without cytosolic proteins to generate superoxide anion was prepared by freezing and thawing a mixture of solubilized membrane and phospholipids. The details of the procedure will be published elsewhere. Briefly, 5 mg/ml PC and and 0.5 mg/ml PA were dissolved in $CHCl_3$. After evaporation of the solvent under nitrogen, the lipid mixture was suspended in a buffer containing 10 mM HEPES pH 7.4, 1 mM $MgCl_2$, 1 mM EGTA, 10 μM GTPγS and 2 mM, and sonicated until the suspension became clear. The lipid suspension was mixed with solubilized membrane (usually, 1 ng PC and 0.1 ng PA/2 × 10^6 cell equivalent membrane fraction/tube), and the mixture was frozen in liquid nitrogen and thawed in a water bath at 37°C. The procedure was repeated six times (29). The membrane thus obtained was activated by SDS without addition of cytosolic proteins and generated superoxide anion upon addition of NADPH. In the membrane preparation, p47-*phox* was not detected by immunoblotting and less than 0.1% of the amount of p67-*phox* necessary for *in vitro* activation was present.

Assay of superoxide generation. The SOD inhibitable cytochrome c reduction assay for superoxide generation was performed in 96-well microtiter plates using Microplate Reader (Bio-Rad model 3550) programmed Kinetics Collector 2.0 (20). The reaction mixture contains 1 mM each of EGTA and $MgCl_2$, 10 μM GTPγS, 2 mM KCl, and 150 μM acetylated cytochrome C, cytosol and the solubilized membrane equivalent to 1-2 × 10^6 cells in 10 mM HEPES buffer (pH 7.4). The mixture was incubated at 37°C for 1 min and to this mixture, SDS was added for activation. The concentration of SDS yielding optimal activity was determined by titration prior to each experiment (80-100 μM range). After incubation at 37°C for 2 min, 300 μM NADPH was added to initiate the reaction and the rate of increasing absorbance at 550 nm was determined. Peptides were added to the reaction mixture before or after the addition of SDS. The potential of the peptides to inhibit superoxide generation was quantified by measuring the concentration of peptides that inhibit 50% of the production (IC50) in the absence of peptides.

In some experiments, SOD inhibitable CLA-dependent luminescence was used as a measure for superoxide generation. In the measurement, 150 μM CLA instead of acetylated cytochrome c was added in the reaction mixture described above and luminescence was determined using Biolumat LB 9505 (BERTHOLD) settled at 35°C. The reaction was started by the addition of 300 μM NADPH. The maximum counts of CLA-dependent luminescence had good correlation with the maximum velocity of the generation determined by the cytochrome c reduction method (correlation coefficient $\gamma = 0.98$).

RESULTS

To test if the predicted NADPH binding sites in gp91-*phox* are actually involved in superoxide generation, we have synthesized various peptides corresponding to structurally predicted NADPH binding sites in gp91-*phox* and their vicinity. These peptides were screened for their ability to inhibit NADPH dependent superoxide generation in a cell free system consisting of solubilized membrane and cytosol from human neutrophils. As shown in Table 1, peptides designated as L418 and L441, whose sequences corresponded to residues 418-435 (IC50 = 12 μM) and 441-435 (IC50 = 58 μM) of gp91-*phox,* respectively, were found to inhibit

TABLE 2

Effect of Truncated Peptides of L418 on Superoxide Generation in a Cell-Free System

Name	Sequence	Residues in gp91-*phox*	IC50 (μM)
L418	ILKSVWYKYCNNATNLKL	418-435	10 ± 1.4
	LKSVWYKYCN	419-418	10 ± 1.0
	LKSVWYKY	419-426	30 ± 2.3
	LKSVWYK	419-425	40 ± 1.7
	LKSVWY	419-424	190 ± 18.3
	KSVWYKYCNNA	420-430	35 ± 4.3
	KSVWYKY	420-426	50 ± 5.6
L420	KSVWYK	420-425	40 ± 4.3
	SVWYKY	421-426	85 ± 6.5
	VWYKYCNNATN	422-432	75 ± 2.8
Control	RGVHFIF	559-565	45 ± 3.1
	RGVHAIF		>250

TABLE 3

Effect of Amino Acid Substitution on Inhibitory Activity of L420

Sequence	IC50 (μM)	Sequence	IC50 (μM)
KSVWYK	35	KSVAYK	>200
ASVWYK	35	KSVAYK	>200
KAVWYK	40	KSVWYA	170
KSAWYK	30	KAAAAK	>200

superoxide generation in the system activated with SDS. L418 had the lowest half-inhibitory concentration (IC50).

To know the minimum sequence of L418 essential for inhibition, a series of truncated peptides were synthesized and tested. Amino acid residues were removed as a block or one by one from amino and/or COOH termini. As shown in Table 2, L420, whose sequence corresponds to residues 420-425 was still highly inhibitory whereas removal of lysine residues at either terminus increased the IC50 greatly. We, therefore, conclude that L420 has the minimum essential sequence for inhibition.

We next performed alanine substitutions to test the contribution of each residue of L420 in the activity. As shown in Table 3, substitution of alanine for amino- and COOH-terminus lysine residues increased the IC50 significantly. However, lysine residues at amino- and COOH-termini were not enough for the inhibition, since the peptide which had lysine residues at both termini and alanine residues in middle did not inhibit superoxide generation. Furthermore, we found that substitution of alanine for the central tryptophan or tyrosine residue increased the IC50 significantly. The results indicate that these four amino acid residues in L420 play essential roles for inhibitory activity.

All peptides so far reported to inhibit superoxide generation in a cell free system from neutrophils exhibited their activity only when they were added to the system prior to activation. Such peptides inhibited assembly of activated enzyme complex (21, 28-30). We found, in contrast, that L418, added even after the system was activated, abolished superoxide generation almost immediately (Fig. 1A), indicating that L418 acted on the activated system. This was further confirmed by the experiments using the activated membrane obtained from stimulated neutrophils. As shown in Fig. 1B, L418 added to the activated membrane inhibited superoxide generation in a dose dependent manner. Furthermore, the inhibitory activity of L418 was also demonstrated in the membrane activated *in vitro* without cytosol (Fig. 1C). Essentially the same results were obtained with L420. These results suggest that activated cytochrome b558 is the target of the inhibition.

To know how L418 and L420 interact with cytochrome b558 in membrane, we performed kinetic analysis. Reaction rate was determined as a function of NADPH concentrations and effect of the peptides on the rate was studied. The double reciprocal plots of the results (Fig. 2) show that all lines obtained with different concentration of L418 have identical slopes of Km/Vmax. [^{32}P]NADPH did not bind to L418 or L420 (data not shown). These results indicate that L418 and L420 are *uncompetitive* inhibitors and suggest that the peptides bind directly to activated cytochrome b558 to which NADPH bound. Ki Values for L418 and L420

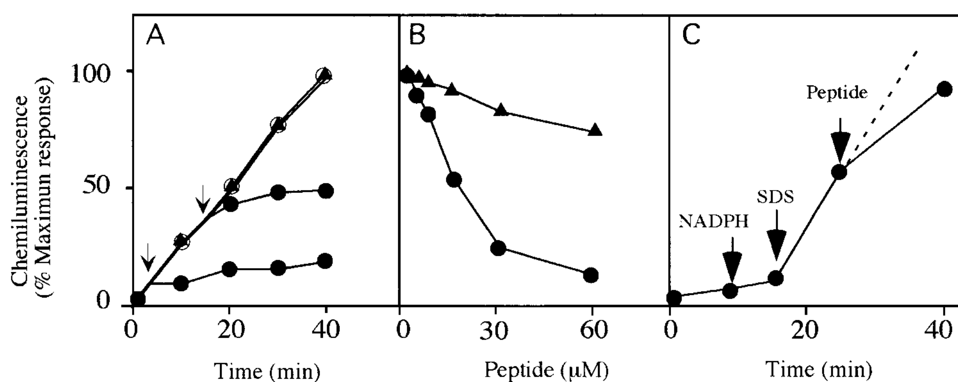

FIG. 1. Effect of peptide L418 on superoxide generation in the cell-free system consisting of membrane and cytosol (A), in activated membrane from stimulated neutrophils (B) or in membrane activated *in vitro* without cytosol (C). (A) To the mixture of solubilized membrane and cytosol, 80 μM SDS was added to activate the system. After 2 min incubation, NADPH was added to start superoxide generation (Time 0), which was measured as CLA-chemiluminescence. L418 (●), L559 (▲) or water (○) was added to the reaction mixture at the time indicated by arrows. To maintain full activity, the same amount of cytosol was supplied at 20 min. (B) To the activated membrane from stimulated neutrophils, NADPH was added to start superoxide generation. After 2 min, indicated concentrations of L418 (●) or L559 (▲) was added, and reduction of light emission of CLA-chemiluminescence was determined. (C) To the frozen and thawed mixture of membrane and lipids, 300 μM NADPH and then 60 μM SDS were added to start superoxide generation, which was monitored as CLA-chemiluminescence. After 10 min of incubation, a half inhibitory concentration of L418 (15 μM) was added. Dotted line indicates the result obtained without addition of the peptide.

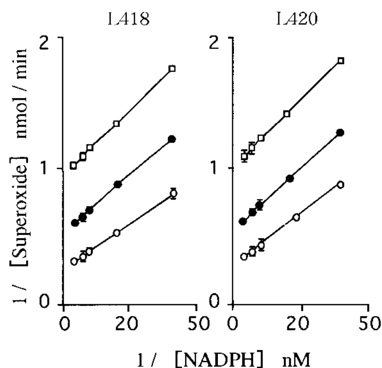

FIG. 2. Kinetic mechanism of inhibition of superoxide generation by L418 (left) and L420 (right) with respect to NADPH. The assay mixture was as described in the text with varying amounts of NADPH as indicated on the abscissa. Either IC50 concentration (●), 2 × IC50 concentration (□) of the peptides or water (○) was added prior to initiating the assay with cytochrome c reduction. The kinetic data are presented in Lineweaver-Burk format. Data are representative of three independent experiments and represent the mean ± standard deviation.

were 11 μM and 36 μM, respectively. Km Values in the presence of these peptides are consistently smaller than the Km value of the uninhibited reaction.

DISCUSSION

We have shown in this paper that peptide whose sequences correspond to residues 418-435, adjacent to one of the predicted NADPH binding sites of the large subunit of cytochrome b558 (gp91-*phox*) inhibited superoxide generation in a cell free assay of neutrophil oxidase activity. The minimum sequence for inhibition was KSVWYK where lysine residues at both termini and central tryptophan and tyrosine residues play essential roles. In contrast to peptides previously reported to inhibit assembly of cytochrome b558 and cytoplasmic proteins, the inhibitory peptides described in the present report, L418 and L420, affected both the activated enzyme complex and membrane activated *in vitro* without cytosol. These peptides, therefore, must interact with activated cytochrome b558, which is believed to shuttle electrons from NADPH to molecular oxygen.

Kinetic analysis revealed that L418 and L420 are *uncompetitive* inhibitors for superoxide generation. This means that L418 and L420 combine reversibly with the activated cytochrome b558 to which NADPH associated (enzyme-substrate complex), cause structural distortion of the active site or NADPH binding site, and thereby inhibit electron transfer from NADPH to molecular oxygen. It is interesting that NADPH is more effectively bound to cytochrome in the presence of L418 or L420, since Km values in the presence of these peptides are consistently smaller than the Km value of the uninhibited reaction.

Among the peptides tested, the peptide corresponding to the residues 441-450 (designated as L441) was also found to inhibit superoxide generation in the cell free system with IC50 = 58 μM. This peptide represents a portion of another peptide previously shown to inhibit superoxide generation, (whose sequence corresponded to residues 434-455 of gp91-*phox* and was designated as peptide No. 9); the IC50 value of the latter peptide was shown to be 25-75 μM (21). It is, therefore, likely that L441 and peptide No. 9 have the similar or identical inhibitory mechanism. In fact, L441 and No. 9 exhibited *irreversible* inhibition (unpublished observation). The results coincide well with the fact that these peptides inhibited superoxide generation by inhibiting association of cytoplasmic components with cytochrome b558 in the membrane (21).

Although further studies are needed to clarify structure-activity relationship of NADPH binding sites of gp91-*phox,* the finding of the *uncompetitive* inhibitory peptides, which seems to cause structural distortion of a NADPH binding site, should provide a new tool for analysis of function of cytochrome b558.

ACKNOWLEDGMENTS

We thank Dr. Philip M. Murphy for critical reading of the manuscript and Martin Dahl for his advice on English expression. This work is partially supported by a Grant in Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture.

REFERENCES

1. Babior, B. M. (1992) *Adv. Enzymol.* **65,** 49–95.
2. Schrenzel, J., Serrande, L., Banfi, B., Nusse, O., Fouyouzi, R., Lew, D. P., Demaurex, N., and Krause, K. H. (1998) *Nature* **392** (6677), 734–737.
3. Kobayashi, S., Imajoh-Ohmi, S., Nakamura, M., and Kanegasaki, S. (1990) *Blood* **75,** 458–461.
4. Nakamura, M., Baxter, C. R., and Masters, B. S. (1981) *Biochem. Biophys. Res. Commun.* **98,** 743–751.
5. Makino, R., Tanaka, T., Iizuka, T., and Kanegasaki, S. (1986) *J. Biol. Chem.* **261,** 11444–11447.
6. Babior, B. M. (1978) *N. Engl. J. Med.* **298,** 659–668.
7. Holms, B., Page, A. R., and Good, R. A. (1967) *J. Clin. Invest.* **46,** 1422–1432.
8. Roos, D., Boer, D. M., Kuribayashi, F., Meischl, C., Weening, R. S., Segal, A. W., Ahlin, A., Nemet, K., Hossle, J. P., Bernatowska-Matuszkiewicz, E., and Middleton-Price, H. (1996) *Blood* **87,** 1663–1680.
9. Clark, R. A., Volpp, B. D., Leidal, K. G., and Nauseef, W. M. (1990) *J. Clin. Invest.* **85,** 714–721.
10. Tyagi, S. R., Neckelmann, N., Uhlinger, D. J., Burnham, D. N., and Lambeth, J. D. (1992) *Biochemistry* **31,** 2765–2774.
11. Parkos, C. A., Allen, R. A., Cochrane, C. G., and Jesaitis, A. J. (1987) *J. Clin. Invest.* **80,** 732–742.
12. Segal, A. W., West, I., Weintjes, F., Nugent, J. H., Chavan, A. J., Haley, B., Garacia, R. C., Rosen, H., and Scrace, G. (1992) *Biochem. J.* **284,** 781–788.

13. Sumimoto, H., Sakamoto, N., Nozaki, M., Sakaki, Y., Takeshige, K., and Minakami, S. (1992) *Biochem. Biophys. Res. Commun.* **186,** 1368–1375.
14. Rotrosen, D., Yeug, T. L., Leto, T. L., Malech, H. L., and Kwong, C. H. (1992) *Science* **256,** 1459–1462.
15. Doussiere, J., Brandolin, G., Derrien, V., and Vignais, P. V. (1993) *Biochemistry* **32,** 8880–8887.
16. Koshkin, V., and Pick, E. (1993) *FEBS Lett.* **338,** 57–62.
17. Koshkin, V., and Pick, E. (1994) *FEBS Lett.* **327,** 285–289.
18. Rotrosen, D., Kleinberg, M. E., Nunoi, H., Leto, T., Gallin, J. I., and Malech, H. L. (1990) *J. Biol. Chem.* **265,** 19910–19915.
19. Nakanishi, A., Imajoh-Ohmi, S., Fujinawa, T., Kikuti, H., and Kanegasaki, S. (1992) *J. Biol. Chem.* **267,** 19072–19072.
20. Park, M-Y., Imajoh-Ohmi, S., Nunoi, H., and Kanegasaki, S. (1994) *Biochem. Biophys. Res. Commun.* **204,** 924–929.
21. Park, M-Y., Imajoh-Ohmi, S., Nunoi, H., and Kanegasaki, S. (1997) *Biochem. Biophys. Res. Commun.* **234,** 531–536.
22. Kleinberg, M. E., Malech, H. L., and Rotrosen, D. (1990) *J. Biol. Chem.* **265,** 15577–15583.
23. Uhlinger, D. J., Tyagi, S. R., and Lambeth, J. D. (1995) *Biochemistry* **34,** 524–527.
24. Kleinberg, M. E., Mital, D., Rotrosen, D., and Malech, H. L. (1992) *Biochemistry* **31.**
25. Park, J. W., Ma, M., Ruedi, J. M., Smith, R. M., and Babior, B. M. (1992) *J. Biol. Chem.* **267,** 17327–17332.
26. Imajoh-Ohmi, S., Tokia, K., Ochiai, M., Nakamura, M., and Kanegasaki, S. (1992) *J. Biol. Chem.* **267,** 180–184.
27. Miki, T., Yoshida, L. S., and Kakimuma, K. (1992) *J. Biol. Chem.* **267,** 18695.
28. Park, J. W. (1996) *Biochem. Biophys. Res. Commun.* **220,** 31–35.
29. Kanegasaki, S., and Jann, K. (1979) *Eur. J. Biochem.* **95,** 287–293.