Regulation of Neutrophil Superoxide by Antichymotrypsin-Chymotrypsin Complexes*

(Received for publication, October 28, 1991)

Mindy G. Schuster†, Philip M. Enriquez‡, Patrick Curran†, Barry S. Cooperman§, and Harvey Rubin†‡

From the †Department of Medicine, Division of Infectious Diseases and the ‡Department of Chemistry, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104

The ability of neutrophils to generate free radicals is a crucial component of host defense (Babiour, B. M. (1978) N. Engl. J. Med. 298, 659–666, 721–725). Neutrophil oxidants, however, can cause significant host tissue destruction (Weiss, S. J. (1989) N. Engl. J. Med. 320, 365–376), and the regulation of free radical production is not well understood. We have previously shown that recombinant antichymotrypsin (rACT), a serine protease inhibitor, inhibits superoxide production in intact neutrophils (Kilpatrick, L., Johnson, J. L., Nickbarg, E. B., Wang, Z., Clifford, T. F., Banach, M., Cooperman, B. S., Douglas, S. D., and Rubin, H. (1991) J. Immunol. 146, 2388–2393). Using a cell-free NADPH oxidase preparation, we now demonstrate that rACT alone has no effect on superoxide production and that antichymotrypsin-chymotrypsin (rACT-CT) complexes are required to inhibit superoxide, suggesting that neutrophil chymotrypsin-like proteases produce conformational changes in ACT, allowing it to become active in regulating superoxide production. Additionally, we have identified NADPH oxidase itself as the target for rACT-CT and have demonstrated that rACT-CT interferes specifically with activation of the NADPH oxidase without changing the kinetic parameters of the oxidase reaction.

In the present study, we explore the mechanism of inhibition of superoxide formation by examining the effect of rACT and the products of the complexation of ACT with chymotrypsin on the activation and turnover of NADPH oxidase in a cell-free system. To study the effect of antichymotrypsin-chymotrypsin complexes on the activation of the respiratory burst oxidase, we employed a model proposed by Babiour et al. (10) for the activation of dormant oxidase to fully active NADPH oxidase.

During the acute phase response to infection and tissue injury, stimulated neutrophils undergo a respiratory burst, producing superoxide radicals via NADPH oxidase. Upon stimulation, the normally dormant NADPH oxidase is assembled from cytosolic and membrane-bound components. Superoxide free radicals, as well as HO₂⁻, OH⁻, and HOCl formed in subsequent reactions are important in the defense against microorganisms (1–2) but can also cause significant damage to host tissue and have been implicated in disorders such as respiratory distress syndrome, myocardial reperfusion damage and rheumatoid arthritis (3–5). Naturally occurring antioxidants such as superoxide dismutase and vitamin E play a role once free radicals have been produced, but little is known about the control of the actual production of free radicals during inflammation.

In addition to the generation of free radicals, stimulated neutrophils release powerful proteases such as elastase and cathepsin G, a chymotrypsin-like enzyme. The serine protease inhibitors antichymotrypsin and α1-protease inhibitor increase dramatically during the acute phase response (7) and are considered to play an important role in mediating the inflammatory response by neutralizing the effects of cathepsin G and elastase respectively. Additional biological functions have been attributed specifically to the complex of the serpin with its target enzyme, and include chemoattractant activity (8) and recognition by membrane receptors (9). Recently, we have shown that in addition to its well known role in inactivating cathepsin G, antichymotrypsin can inhibit the formation of superoxide by stimulated neutrophils (6).

In the present study, we explore the mechanism of inhibition of superoxide formation by examining the effect of ACT and the products of the complexation of ACT with chymotrypsin on the activation and turnover of NADPH oxidase in a cell-free system. To study the effect of antichymotrypsin-chymotrypsin complexes on the activation of the respiratory burst oxidase, we employed a model proposed by Babiour et al. (10) for the activation of dormant oxidase to fully active NADPH oxidase.

\[
\begin{align*}
M + S & \rightarrow M.S \quad k \\
M.S + C & \rightarrow M.S + C_2 \quad M.S + C_3 \quad M.S + C_4
\end{align*}
\]

where M is a membrane component and S is a stabilizing cytosolic component. C₀ and C₂ are cytosolic components that when added to M.S⁺, the immediate precursor of the NADPH oxidase, in a process that requires arachidonic acid or sodium dodecyl sulfate and Mg²⁺, enables the NADPH oxidase to become catalytically active. Both high and low affinity complexes catalyze NADPH-dependent formation of superoxide. k is the first-order rate constant for the rate-limiting step in the activation.

EXPERIMENTAL PROCEDURES

Reagents—Recombinant human antichymotrypsin was purified as previously described (11). Bovine chymotrypsin, bovine erythrocyte superoxide dismutase, ferricytochrome c, GTPyS, ATP, succinyl-

*This work was supported by H & Q Life Sciences, the Garchik Family Fund, and the United States Department of the Navy. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: Dept. of Medicine, Division of Infectious Diseases, University of Pennsylvania School of Medicine, 350 Johnson Pavilion, Philadelphia, PA 19104. Tel.: 213-662-4767; Fax: 213-349-5111.

†‡ The abbreviations used are: GTPyS, guanosine 5'-O-(3-thiotriphosphate); CT, chymotrypsin; ACT, antichymotrypsin; rACT, recombinant antichymotrypsin; PIPES, 1,4-piperazine-dyeacidulfonic acid; EGTA, ethylenediaminetetraacetic acid.
Antichymotrypsin-Chymotrypsin Complexes Inhibit Superoxide

Effect of rACT-CT Added after Activation of NADPH Oxidase — To separate the effect of rACT on activation versus turnover of NADPH oxidase, rACT-CT was added after activation had occurred. Complete activation of the NADPH oxidase was identified as the linear rate of superoxide production following a lag during which superoxide formation gradually accelerates. When rACT-CT complex was added after activation had already occurred (turnover phase), no effect on superoxide formation was seen (Fig. 1C).

Control Experiments — The results of several experiments indicate that the observed inhibition is due to a specific effect of the rACT-CT complex on NADPH-dependent oxidase activity. First, rACT-CT complexes, formed by titration of CT to a 1:1 stoichiometry with rACT, were shown to have no measurable CT activity (based on the hydrolysis of the specific CT substrate N-succinylalanylalanylprolylphenylalanine p-nitroanilide) before or after addition to the cell-free assay. Although CT enhances superoxide production in the intact neutrophil (15), we observe that CT alone can inactivate the NADPH oxidase in the cell-free system. The assay was sufficiently sensitive to detect CT levels below those inhibiting superoxide production. Second, a 30-kDa cutoff filtrate of rACT-CT (obtained using a Centricon 30 microconcentrator) had no effect on superoxide production, excluding lower molecular weight species, including CT, as the agent responsible for inhibition. Third, the degree of inhibition by rACT-CT was not decreased on addition of excess arachidonic acid (Fig. 1D), ruling out simple sequestration of the activator as the mechanism of inhibition. Additionally, our assays typically contain 10 µM GTPγS, which causes a 2-fold increase in superoxide production (16). However, the percent of NADPH oxidase inhibition resulting from the addition of rACT-CT is the same in the presence or absence of GTPγS (data not shown).

Effect of rACT on Km for NADPH, VE, and k — The Km for NADPH was unaffected by the presence of rACT. VE, and k, the first-order rate constant for the rate-limiting step of the activation reaction were determined in the presence and absence of rACT (Table I). It is clear that the inhibitory effect of rACT-CT is principally on lowering VE, rather than on k or on the Km for NADPH.

Preincubation Experiments — Preincubation of rACT-CT with the cytosolic fraction yielded significantly less inhibition of superoxide generation than that following preincubation of rACT-CT with membrane component alone or membrane together with cytosol (Table II), suggesting that the target for rACT-CT is on the membrane.

DISCUSSION

The results presented above support three important conclusions. First, an ACT-protease complex, rather than ACT alone, is responsible for inhibition of superoxide production. We have shown this directly for the cell-free NADPH oxidase system. By extension, we suggest that in the intact neutrophil, ACT can complex with CT-like enzymes released during stimulation, and that complexes like these are responsible for inhibition of superoxide production. Second, inhibition of superoxide production in the cell-free system indicates that the target for rACT-CT is the NADPH-oxidase itself, and not a component of the cellular signaling pathway. Third, within the kinetic scheme describing activation of the NADPH-oxidase proposed by Babior et al. (10), the simplest explanation for the inhibitory effect of rACT-CT consistent with our results is that it interacts with the membrane to reduce the amount of initially formed M-S complex (the product of step 1).
Anti-chymotrypsin-Chymotrypsin Complexes Inhibit Superoxide

**FIG. 1.** A, representative curves of absorbance at 550 nm ($A_{550}$) versus time with and without rACT or rACT-CT. Cell-free assay was as described under "Experimental Procedures." B, concentration-dependent inhibition of superoxide by rACT-CT. Values represent the mean ± standard error (for 2 μM, n = 3; for 4 μM, n = 6; for 8 μM, n = 23; for 10 μM, n = 6; for 12 μM, n = 8). C, effect of 10 μM rACT-CT added before or after arachidonic acid on superoxide production. Curves represent typical tracings of results repeated at least 10 times. Arrows indicate time of rACT-CT addition. Arachidonic acid was added at zero time in both experiments. D, dependence of the maximal rate of superoxide production on arachidonic acid concentration in the presence (open circles, n = 3) and absence (closed circles, n = 4) of 8 μM rACT-CT (± standard error).

**TABLE I**

$K_m$, $V_{max}$ and $k$ for NADPH-oxidase in the presence and absence of rACT-CT

| Conditions | $K_m$ for NADPH (μM) | $V_{max}$ nmol O$_2$/min/mg particulate protein | $k$ min$^{-1}$ |
|------------|----------------------|-----------------------------------------------|---------------|
| No rACT    | 20.7 ± 1             | 363 ± 69                                      | 0.23 ± 0.06   |
| 8 μM rACT-CT | 20.9 ± 1           | 227 ± 44                                      | 0.27 ± 0.06*  |

* By Student’s t test, $p = 0.02$ compared with control (no rACT-CT).

**TABLE II**

Preincubation of 18 μM ACT-CT complex with either cytosol, membrane, or both for 10 min prior to activation by arachidonic acid

| Preincubation | Inhibition of $O_2$% |
|---------------|----------------------|
| Cytosol + membrane | 67 ± 3               |
| Membrane       | 63 ± 4*              |
| Cytosol        | 41 ± 6*              |

* No statistically significant difference ($p > 0.05$) compared with preincubation with cytosol + membrane.

In the intact neutrophil, superoxide generation could be inhibited by ACT even after activation by fMet-Leu-Phe (8). In the cell-free system, however, rACT-CT had no effect when added after full activation of the NADPH-oxidase by arachidonic acid (Fig. 1C). These results are analogous to those seen with N-ethylmaleimide, which inhibited continued production of superoxide in the whole neutrophil but not in the preactivated cell-free system (17). The N-ethylmaleimide results have been interpreted as indicating the existence of an intrinsic, rapid deactivation step and continuously replenished pool of active oxidase that are present in intact neutrophils but not in the cell-free system (17). Consequently, effects measured on intact neutrophils cannot distinguish between activation and turnover of the NADPH-oxidase. The observation that rACT-CT does not abort superoxide production in the preactivated cell-free system confirms that the complex is not a free radical scavenger and demonstrates that rACT-CT specifically inhibits activation of the NADPH-oxidase and not its turnover.

The studies reported here, along with earlier work on the intact neutrophil (6) indicate a potentially important biological role for ACT in regulation of the inflammatory response. ACT normally circulates at approximately 4 μM, but shortly after an inflammatory stimulus the concentration can increase by a factor of 5 (7). During inflammation, there is a disruption of the dynamic equilibrium between proteases and...
their inhibitors (7) that can result in significant tissue damage from neutrophil proteases and free radicals (3–5). Interestingly, oxidation of methionine in the P1 position of α1-protease inhibitor leads to inactivation of this serpin, preventing it from neutralizing neutrophil proteases (7). During conditions associated with neutrophil stimulation, neutrophil proteases released locally might complex with ACT, thus limiting the extent of host damage and preventing oxidative inactivation of other protective serpins by suppressing free radical production. The well known biological activities of serpins include regulation of complement, fibrinolytic and thrombolytic cascades, and inactivation of certain cell-associated proteases (7). Additional biological functions attributed specifically to the serpin-enzyme complex (18) include chemotactant activity (8) and recognition by membrane receptors (9). Animal studies examining the effects of free radical scavengers on diseases where free radicals are implicated in the pathogenesis have yielded conflicting results. The ability to control the actual production of neutrophil free radicals with therapeutic agents, based on the serpin-enzyme complex, might offer promise in diseases such as rheumatoid arthritis, adult respiratory distress syndrome, reperfusion injury, and conditions associated with aging, where free radicals have been implicated in the mechanism of tissue destruction (19–20).

Acknowledgments—We thank Laurie Kilpatrick and Norman B. Schechter for their critical review of the manuscript and Martin Pring for his assistance in computer curve fitting.

REFERENCES
1. Babior, B. M. (1978) N. Engl. J. Med. 298, 659–668, 721–725
2. Johnston, R. B., Jr., and Baehner, R. L. (1971) Pediatrics 48, 700–739
3. Henson, P. M., and Johnston, R. B. (1987) J. Clin. Invest. 79, 669–674
4. Malech, H. L., and Gallin, J. (1987) N. Engl. J. Med. 311, 687–694
5. Weiss, S. J. (1989) N. Engl. J. Med. 320, 365–376
6. Kilpatrick, L., Johnson, J. L., Nickbarg, E. B., Wang, Z., Clifford, T. F., Banach, M., Cooperman, B. S., Douglas, S. D., and Rubin, H. (1991) J Immunol. 146, 2388–2393
7. Travis, J., and Salvesen, G. S. (1983) Annu. Rev. Biochem. 52, 655–709
8. Banda, M. J., Rice, A. G., Griffin, G. L., and Senior, R. M. (1988) J. Exp. Med. 167, 1608–1615
9. Perlmutter, D. H., Glover, G., Meheray, R., Schasteen, C., and Fallon, R. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3785–3787
10. Babior, B. M., Kuver, R., and Curnutte, J. T. (1988) J. Biol. Chem. 263, 1713–1718
11. Robin, H., Wang, Z., Nickbarg, E. B., McLarney, S., Naidoo, N., Schoenberger, O. L., Johnson, J. L., and Cooperman, B. S. (1990) J. Biol. Chem. 265, 1199–1207
12. Gabig, T. G., and Lefker, B. (1986) Methods Enzymol. 132, 335–344
13. Gabig, T. G., Eklund, E. A., Potter, G. B., and Dykes, J. R., II (1990) J. Immunol. 145, 945–951
14. Smith, P. K., Krohn, R. L., Hermanson, G. T., Mallia, A. K., Gartner, P. H., Provenzano, M. D., Frigimato, E. K., Goerke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–80
15. Kusner, D. J., and King, C. H. (1989) J. Immunol. 143, 1696–1702
16. Gabig, T. G., English, D., Akard, L. P., and Schell, M. (1987) J. Biol. Chem. 262, 1685–1690
17. Akard, L. P., English, D., and Gabig, T. G. (1988) Blood 72, 322–327
18. Kurdowska, A., and Travis, J. (1990) J. Biol. Chem. 265, 21023–21026
19. Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M., and Harmon, D. (1987) Ann. Intern. Med. 107, 526–545
20. Southern, P. A., and Powis, G. (1988) Mayo Clin. Proc. 63, 390–408