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Oxidative inactivation of myeloperoxidase released from human neutrophils

Steven W. EDWARDS,* Heather L. NURCOMBE* and C. Anthony HART†
Departments of *Biochemistry and †Medical Microbiology, University of Liverpool, P.O. Box 147, Liverpool L69 3BX, U.K.

Within 1 min of stimulation of human neutrophils by the chemotactic peptide (*N*-formyl-L-methionyl-L-leucyl-L-phenylalanine) plus cytochalasin B, myeloperoxidase (together with other granule enzymes) was secreted and detected extracellularly. In contrast with the other granule constituents assayed (vitamin B_{12}-binding protein and β-glucuronidase), the activity of released myeloperoxidase rapidly decreased, so that, by 10 min after stimulation, only about 5% of the total cellular activity was detected. This inactivation was shown to be dependent on oxidant generation during the respiratory burst, since inactivation was not observed (*a*) after stimulation of anaerobic suspensions or (*b*) after release from neutrophils from a patient with chronic granulomatous disease; purified myeloperoxidase was rapidly inactivated after incubation with H_2O_2, presumably owing to the formation of an inactive enzyme–H_2O_2 complex. These results show that experiments designed to assess the role of myeloperoxidase in neutrophil functions which utilize assays based on peroxidase activity will grossly underestimate this enzyme if oxidant generation during the respiratory burst has also been activated.

INTRODUCTION

In order to carry out their crucial role in host defence, polymorphonuclear leucocytes (neutrophils) possess a number of cytotoxic enzymes and associated pathways which can be utilized for cell killing. One such cytotoxic system is provided by myeloperoxidase, a haemoprotein located within azurophilic granules (Klebanoff & Clark, 1978). During phagocytosis, azurophilic granules fuse with the newly formed phagosome and thus myeloperoxidase (together with other granule enzymes) is targeted towards the ingested bacterium by the process of degranulation (Karnovsky & Bolis, 1982). Myeloperoxidase may then react with H_2O_2 generated during the respiratory burst (Babior, 1978), together with a halide (usually Cl^-) to generate HOCl and other related compounds with wide biological activity (Stelmaszynska & Zgliczynski, 1974; Harrison & Schultz, 1976; Winterbourn, 1985). Indeed, the myeloperoxidase–H_2O_2–halide system has been demonstrated to kill effectively many types of micro-organisms and tumour cells *in vitro*, but its potency *in vivo* depends on activation of H_2O_2 generation during the respiratory burst and degranulation.

In addition to its role in cytotoxicity, myeloperoxidase has also been shown to mediate a number of other important inflammatory processes. Such functions include both activation and inactivation of secreted neutrophil proteins (Clark & Borregaard, 1985; Weiss *et al.*, 1985; Peppin & Weiss, 1986), inactivation of toxins and other inflammatory mediators (Klebanoff & Clark, 1978), scavenging of $OH^{\cdot}$ (hydroxyl radical) (Winterbourn, 1986) and termination of the respiratory burst itself (Rosen & Klebanoff, 1976; Cech *et al.*, 1979; Dri *et al.*, 1985; Edwards & Swan, 1986). Since on activation by certain stimuli neutrophils may secrete myeloperoxidase concomitant with oxidant generation, an extracellular myeloperoxidase–H_2O_2–halide system may also evoke damage to host tissues during an inflammatory response.

In view of the important role of released myeloperoxidase during the inflammation and its potential to severely damage host tissues in inflammatory diseases such as rheumatoid arthritis, the aim of the present work was to establish the kinetics of secretion and extracellular function of this enzyme under conditions whereby oxidant generation during the respiratory burst was carefully regulated.

EXPERIMENTAL

Preparation of neutrophils

Human polymorphonuclear leucocytes (neutrophils) were isolated from heparinized venous blood from healthy volunteers, by using a combined dextran/Ficoll–paque separation procedure (Edwards & Swan, 1986) or by utilizing Mono-Poly Resolving Medium (M-PRM; Flow Laboratories) as described in the manufacturer's instructions. After purification, cells were suspended in buffer [containing (mM): NaCl, 120; KCl, 4.8; KH_2PO_4, 1.2; $CaCl_2$, 1.3; $MgSO_4$, 1.2; Hepes, 25 (pH 7.4) and 0.1% bovine serum albumin] counted on a Fuchs–Rosenthal haemocytometer slide and used within 4 h of preparation.

Analytical methods

Suspensions of neutrophils [usually (0.5–1) × 10^7 cells/ml] were incubated at 37 °C in a stirred reaction vessel fitted with an oxygen electrode (Edwards *et al.*, 1984). At suitable time intervals after stimulation, samples were

‡ Abbreviations used: $O_2^{-\cdot}$, superoxide radical; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; CGD, chronic granulomatous disease.

removed, centrifuged for 20 s in an MSE Microcentaur centrifuge (10500 *g*), and the supernatants recovered; these were either analysed immediately or stored at −20 °C. Enzyme release after stimulation under anaerobic conditions was measured by incubating suspensions under a gas phase of O_2-free N_2 and directly measuring the O_2 partial pressure in the liquid phase with an oxygen electrode (< 0.5 μM-O_2); in these experiments the sampling syringe and collecting tubes were sparged with N_2 before sampling. The activity released after incubation of cells with 0.5% Triton X-100 (v/v, final concn.) for 5 min at 4 °C was taken as 100%.

Human myeloperoxidase was purified from buffy-coat preparations as described by Pember *et al.* (1983); the A_{430}/A_{280} was 0.68. Vitamin B_{12}-binding protein was assayed by the method described by Gottlieb *et al.* (1965), whereas myeloperoxidase and β-glucuronidase activities were measured as described previously (Bergmeyer, 1974), with guaiacol and *p*-nitrophenyl β-D-glucuronide, respectively, as substrates.

Chemicals

fMet-Leu-Phe, cytochalasin B, guaiacol, *p*-nitrophenyl β-D-glucuronide and Triton X-100 were obtained from Sigma, whereas cyano[^{57}Co]cobalamin was obtained from Amersham International. All other chemicals were of the highest purity available.

RESULTS

Kinetics of granule enzyme release

After stimulation of neutrophils by fMet-Leu-Phe plus cytochalasin B, maximal activity of myeloperoxidase in supernatants (released from azurophilic granules) was detected within 1 min of stimulation (Fig. 1). After this time, however, the measured activity rapidly decreased, and, within 10 min after stimulation, only about 5% of the total cellular activity was detected. Similarly, the activity of released β-glucuronidase (from azurophilic granules and C-particles) was maximal by approx. 1 min after stimulation, but the activity of this enzyme in supernatants remained constant throughout the course of the experiment. In contrast, the activity of released vitamin B_{12}-binding protein in supernatants gradually increased throughout the sampling time. No further changes in activities were detected over the following 10 min (results not shown).

Effect of O_2 on the activity of released myeloperoxidase

As with myeloperoxidase release, maximal rates of $O_2^{-\cdot}$ and H_2O_2 generation also occur within 1 min of activation of human neutrophils by this stimulus (Edwards & Swan, 1986; Edwards, 1987). Therefore, in order to ascertain whether this decrease in activity of released myeloperoxidase was related to $O_2^{-\cdot}$ or H_2O_2 production, neutrophil suspensions were stimulated under anaerobic conditions so that oxidant generation was prevented. Whereas the maximal level of released activity (within 1 min) was approximately the same as that measured under aerobic conditions, under anaerobic conditions the activity in supernatants did not rapidly decrease, but was maintained (Fig. 2). In the particular experiment shown, small amounts of O_2 were introduced into the suspension halfway through the experiments; upon exposure to O_2 (which then allowed oxidant generation), the activity of released myeloperoxidase rapidly decreased.

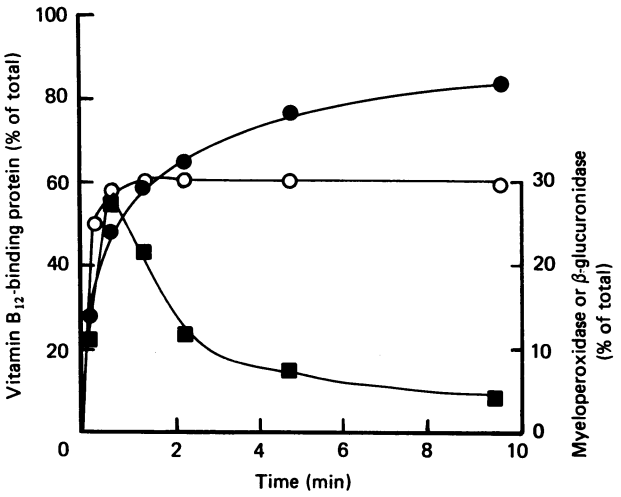

Fig. 1. Kinetics of granule enzyme release from neutrophils

Neutrophil suspensions (10^7 cells/ml in buffer) were incubated with gentle stirring at 37 °C. Before stimulation, portions were removed, centrifuged as described in the Experimental section, and recovered supernatants were assayed; in such unstimulated suspensions, enzyme release was undetectable. Portions of suspensions were then incubated with 0.5% Triton X-100 for 5 min at 4 °C and released activity was taken as 100%. At zero time suspensions were stimulated by the addition of 1 μM-fMet-Leu-Phe plus cytochalasin B (1 μg/ml) (final concns.), samples removed and released enzyme activities measured in supernatants. Data shown are a typical kinetic profile obtained from at least five separate experiments showing (●) vitamin B_{12}-binding protein, (○) β-glucuronidase and (■) myeloperoxidase. The total activities (100%) were as follows: myeloperoxidase, 11.3 munits/10^7 cells (±3.4, n = 12); β-glucuronidase, 1.9 munits/10^7 cells (±0.5, n = 12); vitamin B_{12}-binding protein, 2.16 ng bound/10^7 cells (n = 8).

Myeloperoxidase release from neutrophils from a CGD patient

Neutrophils from a CGD patient exhibited maximal rates of O_2 generation on exposure to fMet-Leu-Phe plus cytochalasin B of 0.5 nmol/min per 10^6 cells compared with control values of 2.4±0.54 (n = 18) nmol/min per 10^6 cells. Myeloperoxidase release from neutrophils from this patient was again maximal within 1 min after stimulation (Fig. 3), but activity was maintained at this level throughout the course of the experiment, again confirming the role of neutrophil-derived oxidants in the decrease in activity observed in control neutrophils.

Inactivation of myeloperoxidase by H_2O_2

In order to confirm that the decrease in activity of released myeloperoxidase was due to its interaction with H_2O_2 generated during the respiratory burst, the peroxidative activity of the purified enzyme was measured after incubation for 10 min at 37 °C with increasing concentrations of H_2O_2: the activity of the purified enzyme was over 90% inhibited by exposure to 0.5 mM-

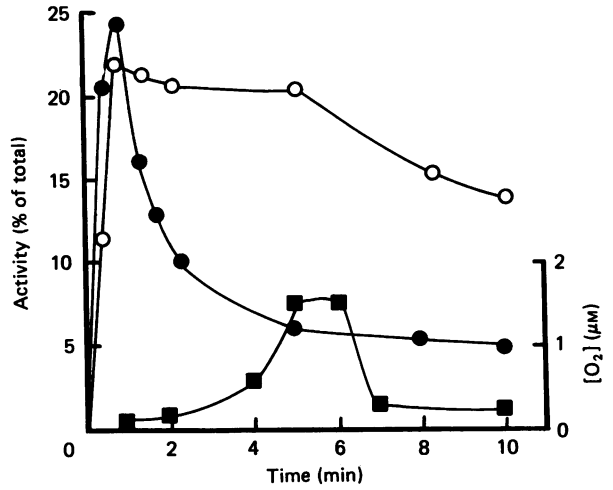

Fig. 2. O_2-dependent inactivation of released myeloperoxidase

Experimental details were as described in the legend to Fig. 1, showing myeloperoxidase activity in supernatants released from neutrophils activated under aerobic conditions (●). Myeloperoxidase release was then measured in a suspension of neutrophils incubated and activated under similar conditions except that, before activation, the suspension was made anaerobic for 10 min by equilibration with N_2 (○). In this experiment traces of O_2 were introduced into the suspension (and measured by using an oxygen electrode, ■) approximately halfway through the experiment. The result shown is typical for three separate experiments.

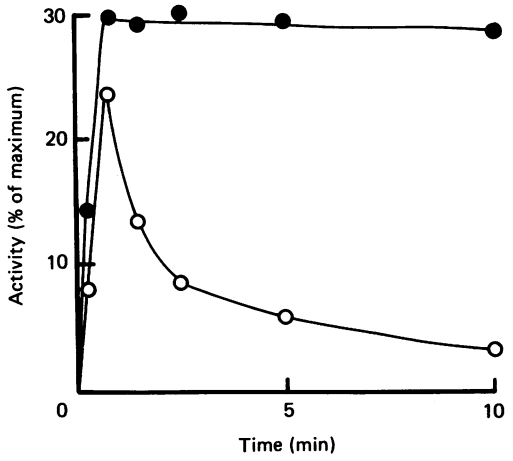

Fig. 3. Myeloperoxidase release from neutrophils from a patient with CGD

Neutrophils were incubated and activated as described in the legend to Fig. 1. ○, Activity of myeloperoxidase released from control neutrophils; ●, activity of myeloperoxidase released from neutrophils of the CGD patient. The result is typical for at least three separate experiments.

H_2O_2, with half-maximal inhibition observed at 10–15 μM (Fig. 4). No variation in H_2O_2-dependent inactivation of myeloperoxidase was observed in the presence of 1% or 10% (v/v) bovine serum.

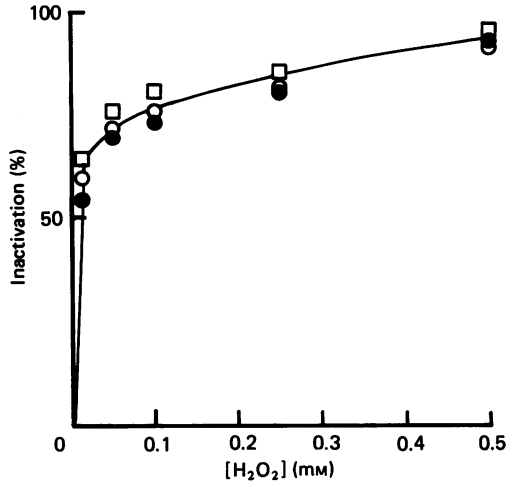

Fig. 4. Inactivation of purified myeloperoxidase by H_2O_2

Purified myeloperoxidase (10 nM) was incubated with various concentrations of H_2O_2, in the presence or absence of bovine serum. After 10 min incubation at 37 °C, peroxidase activity was measured by the guaiacol method, as described in the Experimental section. Myeloperoxidase was incubated with H_2O_2 in: ●, the absence of exogenous proteins; ○, in the presence of 1% bovine serum; ■, in the presence of 10% bovine serum.

DISCUSSION

The results presented here show that, when human neutrophils are stimulated by fMet-Leu-Phe plus cytochalasin B, myeloperoxidase (together with other granule enzymes) is rapidly secreted and detected extracellularly. However, in contrast with vitamin B_{12}-binding protein (whose extracellular activity slowly increased, owing to continued secretion) and β-glucuronidase (whose extracellular activity remained constant from 1 min after stimulation), the activity of released myeloperoxidase rapidly decreased so that, by 10 min after stimulation, only 5% of the total cellular activity was detectable. Although there are a number of reports of cytochalasin B-treated cells actively secreting myeloperoxidase (e.g. Hallett & Campbell, 1983), none have described such a rapid inactivation of the released enzyme. A decrease in total cellular activity of myeloperoxidase was reported after phagocytosis of bacterial by neutrophils (Bradley *et al.*, 1982), but the molecular basis for this inactivation was not described.

In addition to the enzymes measured in the present study, neutrophil granules also contain a number of proteinases that are potentially capable of activating/inactivating released enzymes, thus regulating their extracellular activity. However, we rule out the possibility that inactivation of released myeloperoxidase was due to such proteinase activity. Firstly, myeloperoxidase inactivation only occurred when neutrophils were stimulated to degranulate in the presence of O_2; oxidant generation during the respiratory burst is crucially dependent on O_2 supply (Edwards *et al.*, 1984). Secondly, myeloperoxidase inactivation was not observed after stimulation of neutrophils from a CGD patient. Thirdly, the inactivation of myeloperoxidase could be mimicked *in vitro* by incubating the purified enzyme with H_2O_2; we

have shown that the rates of O_2 and H_2O_2 generation are maximal when myeloperoxidase is released (i.e. 1–2 min after stimulation; Edwards & Swan, 1986; Edwards, 1987). This inactivation was not prevented by the addition of exogenous proteins, so it is unlikely that serum proteins protect myeloperoxidase from H_2O_2 *in vivo*. Thus we conclude that the rapid inactivation of released myeloperoxidase is due to the formation of an inactive myeloperoxidase–H_2O_2 complex (Winterbourn *et al.*, 1985), and we have previously shown that complex II formation occurs when neutrophils are activated under the conditions employed in the present study (Edwards & Lloyd, 1986). It is noteworthy that whereas maximal rates of oxidant generation by neutrophils from the CGD patient were about 20% of normal, H_2O_2-dependent inactivation was not observed, making it unlikely that myeloperoxidase provided effective cytotoxic protection in this patient.

The role of myeloperoxidase during inflammation and in inflammatory diseases is equivocal, but the results prevented here may enable us to distinguish between the release of this enzyme from lysed neutrophils and its active secretion concomitant with H_2O_2 generation to generate HOCl and related compounds extracellularly; in the latter case the enzyme will be present in inflammatory fluids in an inactive form and only detected by immunological assays (e.g. Olsen *et al.*, 1986). Furthermore, experiments designed to examine the role of myeloperoxidase in neutrophil functions utilizing assays based on its peroxidative activity (e.g using guaiacol or *o*-dianisidine) will inevitably grossly underestimate the enzyme if the respiratory burst has also been activated. One therefore needs to extend these observations to search for immunodetectable myeloperoxidase in inflammatory fluids in order to evaluate the role of this enzyme *in vivo*.

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