Accelerating Effect of Glutathione on Hydroxylation of Phenylalanine by Stimulated Polymorphonuclear Leukocytes

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Sulfhydryl compounds significantly accelerated the hydroxylation of phenylalanine by stimulated polymorphonuclear leukocytes. The reduced form of glutathione (G-SH) was most effective. The hydroxylation reaction in the presence of G-SH was largely prevented by superoxide dismutase and hydroxyl radical scavengers. The results suggest that a much faster production of hydroxyl radical may occur in a reaction mixture containing both G-SH and stimulated polymorphonuclear leukocytes than in that containing stimulated polymorphonuclear leukocytes alone.

Keywords polymorphonuclear leukocyte; oxygen radical; glutathione; phenylalanine; *o*-tyrosine; *m*-tyrosine

Upon exposure to appropriate stimuli, polymorphonuclear leukocytes (PMNs) undergo what is termed "a respiratory burst", whose purpose is the production of microbicidal oxidizing species. This respiratory burst results in the production of large quantities of superoxide (O_2^-) and H_2O_2, which may then interact to form hydroxyl radical ($OH^\cdot$).[1-3] The $OH^\cdot$ may take a significant part in the bactericidal activity of PMNs.[1] We previously demonstrated using phenylalanine as $OH^\cdot$ trapping agent and high performance liquid chromatography (HPLC) that the generation of $OH^\cdot$ occurs during the respiratory burst of PMNs.[4] During studies on the effectors modifying $OH^\cdot$ generation of PMNs by the $OH^\cdot$ trapping method using phenylalanine and HPLC,[4] we obtained the interesting findings that the reduced form of glutathione (G-SH) accelerates the production of $OH^\cdot$ by stimulated PMNs. This paper describes this accelerating effect of G-SH on hydroxylation of phenylalanine by PMNs.

Materials and Methods

Phenylalanine, penicillamine, phorbor miristate acetate (PMA), zymosan, sodium myristate, formyl-methionyl-leucyl-phenylalanine (FMLP), bovine serum albumin, cytochrome c (Type III), superoxide dismutase (SOD, from bovine erythrocytes, 3000 unit/mg), and catalase (from bovine liver, 44000 unit/mg) were obtained from Sigma Chemical Co. G-SH, the oxidized form of glutathione (G-S-S-G), cysteine, dithiothreitol, 2-mercaptoethanol, sodium benzoate, and mannitol were from Wako Pure Chemicals. Zymosan was opsonized with guinea pig serum.

PMNs were obtained from casein-induced peritoneal exudates of female guinea pigs of Hartley strain as described previously.[5] Reaction mixtures containing phenylalanine (1 mM), PMNs (10^7 cells), glucose (5 mM), $CaCl_2$ (0.5 mM), and PMA (0.16 μM) in the presence and absence of sulfhydryl (SH) compounds and radical scavengers, in 1.0 ml of Krebs Ringel phosphate buffer were incubated at 37 °C with shaking. After incubation for indicated times, 200 μl of trichloroacetic acid (6 M) was added. After centrifugation, 200 μl of the supernatants was directly injected into HPLC. The *o*- and *m*-tyrosines derived from phenylalanine were measured by HPLC under the conditions described previously.[6]

O_2^- production by PMNs was measured on the basis of ferricytochrome c reduction by the anion produced.[7]

Results

Accelerating Effect of G-SH on Hydroxylation of Phenylalanine by PMA-Stimulated PMNs Figure 1 shows chromatograms of the reaction mixtures obtained by reaction of phenylalanine with PMA-stimulated PMNs in the presence and absence of G-SH. A larger amount of *m*- and *o*-tyrosine was detected in the reaction mixture with G-SH than that detected in the reaction mixture without G-SH. *p*-Tyrosine could not be measured because of the large amount of *p*-tyrosine already in the cells. In the following experiments, the amount of hydroxylated phenylalanine is expressed as the sum of *o*- and *m*-tyrosines. Significant production of tyrosines was not observed by G-SH alone.

Figure 2 shows the time course of the production of tyrosine (*o*- and *m*-) by PMA-stimulated PMNs in the presence and absence of G-SH (5 mM). Significant acceleration of tyrosine production by G-SH was observed.

This accelerating effect of G-SH was also observed when FMLP (0.1 mM), myristate (0.2 mM), NaF (20 mM) or opsonized zymosan (4 mg/ml) was used as stimulus. Under the same conditions as those described in Fig. 2, except for a stimulus other than PMA, G-SH (5 mM) increased tyrosine production by FMLP-, myristate-, NaF- and opsonized zymosan-stimulated PMNs at 30 min of incubation by approximately 4.4, 7.3, 7.6 and 3.2 fold, respectively.

The acceleration was clearly concentration-dependent at lower concentrations of G-SH (Fig. 3). With heat-treated PMNs, tyrosine production was not significant even though

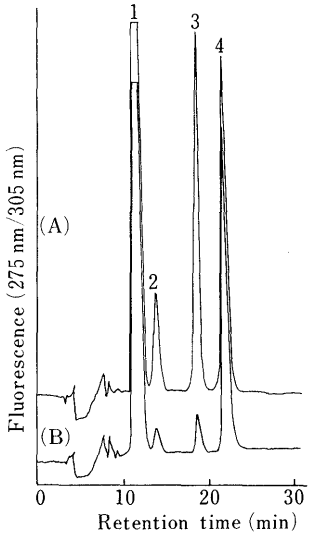

Fig. 1. Chromatogram of the Reaction Mixture

A) The reaction mixture contained phenylalanine, PMNs, glucose, $CaCl_2$, PMA, and G-SH (5 mM) in 1.0 ml of Krebs Ringer phosphate buffer. After 30 min of incubation, the reaction mixture was applied to HPLC as described in Materials and Methods. B) Same as A), except that there was no external addition of G-SH. Peaks 1, 2, 3, and 4 correspond to *p*-tyrosine, *m*-tyrosine, *o*-tyrosine, and phenylalanine, respectively.

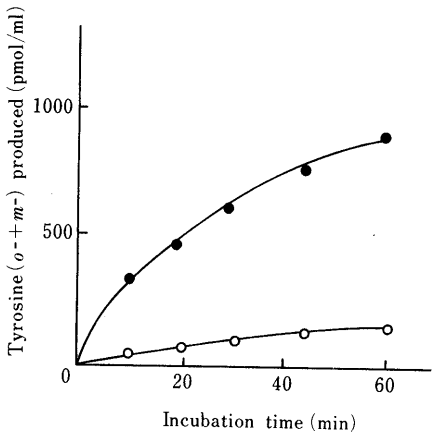

Fig. 2. Time Courses of Tyrosine Production from Phenylalanine by PMA-Stimulated PMNs

Phenylalanine was incubated with PMNs, PMA, glucose, and $CaCl_2$ in the presence (●) and absence (○) of G-SH (5 mM) under the conditions described in Materials and Methods. Samples were incubated for the indicated times, and then assayed as described.

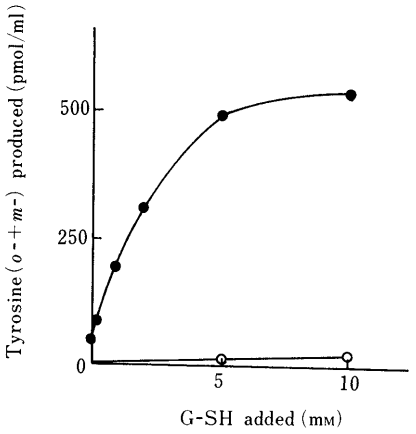

Fig. 3. Effect of G-SH on Tyrosine Production from Phenylalanine by PMA-Stimulated PMNs

Phenylalanine was incubated with PMNs (●) or with heat-treated PMNs (○) in the presence of PMA, glucose, $CaCl_2$ and the indicated concentrations of G-SH under the conditions described in Materials and Methods. Samples were incubated for 30 min and then assayed as described. Heat-treated PMNs were prepared by incubation of PMNs for 5 min in boiling water.

G-SH was present (Fig. 3). These results suggest that the accelerating effect of G-SH may be coupled to O_2^- generation by PMNs.

Effect of various SH compounds on tyrosine production from phenylalanine by PMA-stimulated PMNs is shown in Table I. These various compounds other than G-SH also showed the accelerating effect, though differences in its extent were noted. G-SH was the most effective. There was no significant production of tyrosines by SH compound alone.

Stability of the O_2^--producing ability of PMA-stimulated PMNs was examined in medium with and without G-SH. PMNs (10^7 cells) were incubated with PMA (0.16 μM), glucose (5 mM), and $CaCl_2$ (0.5 mM) in the presence and absence of G-SH (5 mM) in Krebs Ringer phosphate buffer for 5, 30 and 60 min. At the end of each incubation period, the incubated cells were washed twice with the same buffer by centrifugation, and then assayed for O_2^--producing ability as described in Materials and Method. In results, there was no significant difference in O_2^--producing ability between the PMNs incubated with and those incubated

Table I. Effect of Various Sulfhydryl Compounds on Tyrosine Production from Phenylalanine by PMA-Stimulated PMNs

Sulfhydryl compound added	Conc. (mM)	Tyrosine (o-+m-) produced % of control[a]
None (control)	5	100
G-SH	5	895
Cysteine	5	451
Penicillamine	5	665
Dithiothreitol	5	260
2-Mercaptoethanol	5	193
(G-S-S-G	5	108)

Phenylalanine was incubated with PMNs, PMA, glucose, $CaCl_2$, and the specified amount of various sulfhydryl compounds under the conditions described in Materials and Methods. Samples were incubated for 30 min, and then assayed as described in Materials and Methods. *a*) Average of three experiments with the same batch of PMNs.

Table II. Effect of Radical Scavengers on Tyrosine Production from Phenylalanine by PMA-Stimulated PMNs in the Presence of G-SH

Scavenger added	Conc.	Tyrosine (o-+m-) produced % of control[a]
None (control)		100
Sodium benzoate	25 mM	25
Mannitol	100 mM	17
SOD	4 μg/ml	4.4
Catalase	100 μg/ml	49
Bovine serum albumin	100 μg/ml	89

Phenylalanine was incubated with PMNs, PMA, glucose, $CaCl_2$, G-SH (5 mM) and the specified amount of various substances under the conditions described in Materials and Methods. Samples were incubated for 30 min, and then assayed as described. *a*) Average of three experiments with the same batch of PMNs.

without G-SH, though both PMNs preparations gradually lost the O_2^--producing ability with incubation time: O_2^--producing abilities of PMN preparations after 30 and 60 min of incubation were about 83 and 60%, respectively, that of PMNs incubated for 5 min. This suggests that the accelerating effect of G-SH for hydroxylation of phenylalanine by PMA-stimulated PMNs is not due to the stabilization of O_2^--producing ability of PMNs by G-SH.

Effectors Modifying the Hydroxylation of Phenylalanine by PMNs in the Presence of G-SH To obtain evidence as to the nature of the oxidative species in the presence of G-SH, the effect of OH· scavengers on the tyrosine production by PMA-stimulated PMNs was examined (Table II). OH· scavengers, such as benzoate and mannitol, effectively prevented tyrosine production, though they did not significantly affect the O_2^--producing ability of PMA-stimulated PMNs. This may suggest the acceleration of OH· generation by G-SH in the medium containing PMA-stimulated PMNs. The role of O_2^- and H_2O_2 in production of tyrosines in the medium containing PMNs and G-SH was also examined by testing the effect of SOD and catalase (Table II). SOD strongly inhibited the tyrosine production, while catalase was less effective. The inhibitory effect of SOD was greater in medium containing both PMNs and G-SH than that in medium containing PMNs alone: in the former medium, SOD (4 μg/ml) inhibited tyrosine production to 4.4% of control (Table II), whereas in the latter medium, SOD (20 μg/ml) inhibited the production to 33%.[4] On the other hand, catalase (100 μg/ml) showed quite similar inhibitory effects in media both with and without

G-SH: 49% (Table II) and 55%,[4] respectively. These findings suggest that O_2^- may be a main precursor of $OH^\cdot$ in the presence of G-SH.

Discussion

PMNs not only produce O_2^- but also other oxidants such as HOCl, which reacts readily with taurine[8] or dimethylthiourea.[9] We thought there might be a possibility that the above accelerating effect of G-SH was the result not of an increased generation of tyrosines, but rather of a decrease in further oxidation of tyrosines by HOCl. We have observed that taurine (10 mM) and dimethylthiourea (5 mM) did not accelerate the hydroxylation of phenylalanine by PMA-stimulated PMNs, but reduced the hydroxylation of phenylalanine to about 90 and 75% of control, respectively.[12] This fact and the finding that SOD strongly inhibits the hydroxylation of phenylalanine by PMA-stimulated PMNs in the presence of G-SH (Table II), however, appear to indicate that the mechanism of the G-SH accelerating effect would not allow the possibility suggested above.

Van Steveninck *et al.*[10] have shown that *N*-acetylhomocysteinylglycine as a SH compound accelerates hydroxylation of salicylate in a system containing H_2O_2 and Fe^{3+}–ethylenediaminetetraacetic acid (EDTA), and ascribed this to the ability of SH to reduce trace amounts of transition metals. Hydroxylation of phenylalanine by PMA-stimulated PMNs was inhibited by diethylenetriaminepentaacetic acid, a chelating agent, to about 70% of control.[12] This suggests that $OH^\cdot$ production in medium containing PMNs may occur partly as a result of the Haber–Weiss reaction[11] catalyzed by a trace amount of transition metals contaminating the medium, so called metal-catalyzed Haber–Weiss reaction.[11] In our present system containing PMA-stimulated PMNs and G-SH, the accelerating effect of G-SH may also be due to the ability to reduce trace amounts of transition metals, as described by Van Steveninck *et al.*

SH compounds such as G-SH can act as radical scavengers of $OH^\cdot$[13] and O_2^-.[14] In fact, we have observed that hydroxylation of phenylalanine by both systems of hypoxanthine–xanthine oxidase and hypoxanthine–xanthine oxidase–Fe^{3+}–EDTA (0.1 mM) as models of O_2^- production is significantly inhibited by the addition of G-SH (5 mM), though xanthine oxidase activity is not affected.[12] The hydroxylation by the O_2^--producing system of stimulated PMNs, however, was greatly accelerated by G-SH as described above. The mechanism of the accelerating action of G-SH upon hydroxylation of phenylalanine by PMNs thus seems complex.

Although the mechanism of the accelerating effect remains obscure, the present findings may suggest that acceleration of $OH^\cdot$ production by PMNs occurs when G-SH is present in medium containing PMNs, and that the bactericidal ability of PMNs increases in medium containing G-SH.

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