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IMINODIPEPTIDES CONTAINING PROLINE WITH C-TERMINAL AND N-TERMINAL RESIDUES PRIME THE STIMULATION OF HUMAN NEUTROPHIL SUPEROXIDE GENERATION BY fMLP

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Preincubation of human peripheral blood polymophonuclear leukocytes with an iminodipeptide containing proline at the C-terminus and/or N-terminus, Pro-Pro, Gly-Pro, Pro-Gly, Ala-Pro and Pro-Ala, significantly enhanced N-formyl-methionyl-leucyl-phenylalanine-induced superoxide generation in a concentration-dependent manner. The iminodipeptide with C-terminal proline showed higher effect than that of the counterpart with N-terminal proline. These iminodipeptides also enhanced the superoxide generation induced by opsonized zymosan but not that induced by arachidonic acid or phorbol myristate acetate. Tyrosyl phosphorylation of the 45-kDa protein occurred in parallel with the iminodipeptide-dependent enhancement of superoxide generation in neutrophil.

Stimulation of polymorphonuclear leukocytes (PMN) by soluble and particulate stimuli triggers rapid and massive production of superoxide anion (O_2^-), so-called "respiratory burst", which plays an important role in the killing of bacteria and in host tissue damage [1-3]. Various compounds such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), opsonized zymosan (OZ), phorbol 12-myristate 13-acetate (PMA) and arachidonic acid (AA) are known as the stimuli [4]. Although the responsibility of PMN to agonist is low in the basal condition, preincubation of PMN with non-stimulatory concentrations of agonists or some pharmacological agents and hypotonic treatment of the cells accelerate and potentiate the respiratory burst induced by a second stimulus [5-12]. This phenomenon, termed "priming", may account for the exaggerated physiological responses of human peripheral blood polymophonuclear leukocytes (HPPMN). Priming and stimulation of PMN has been proposed to occur through different mechanisms [13, 14] while they still remain to be clarified. Recently, several reports described that various cytokines and hypotonic condition enhanced the tyrosyl phosphorylation of specific proteins in HPPMN in the primed stage, suggesting the

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contribution of tyrosine kinase (TK) to the regulatory mechanism of priming in neutrophils [11, 14-16].

Prolidase (EC. 3, 4, 13, 9) deficiency is a rare autosomal recessive disease characterized by chronic ulcerative dermatitis and mental retardation [17, 18]. It has been known that these patients excrete large amounts of iminodipeptides in the urine, and we recently reported that the iminodipeptides are also increased in serum of the patients by using LC/APCI-MS [19, 20].

In the present study, we examined the effect of various iminodipeptides containing proline at the C-terminus and/or N-terminus on the stimulation-coupled responses of HPPMN.

MATERIALS AND METHODS

Chemicals : NADPH, Ferricytochrome *c* (cyt. *c*), superoxide dismutase, fMLP, zymosan, PMA, AA, amino acids, iminodipeptides and iminotripepides were purchased from Sigma Chemical Co. All other reagents used were of analytical grade and were from Nacalai Tesque Inc.

Isolation of neutrophils : HPPMN were isolated from the peripheral blood of healthy humans by Ficoll-Hypaque (Flow Laboratories) density gradient centrifugation [21] and were washed twice with Krebs-Ringer-phosphate solution (KRP; pH 7.4) [22]. The cells were counted and resuspended in KRP at a concentration of $1x10^8$ cells/ml.

Assay of superoxide generation : The O_2^- generation was assayed by measuring the reduction of cyt. *c* at 37 °C using a dual-beam spectrophotometer (Shimadzu UV-3000) under continuous stirring [14]. Four different compounds were employed as the stimuli of neutrophils; fMLP (12.5 nM), OZ (200 μg/ml), PMA (1 nM) and AA (5 μM). Stock solutions of fMLP, PMA and AA were prepared with ethanol. OZ was prepared according to the method of Nagata and Yamashita [23]. The standard assay mixture consisted of $1x10^6$ cells/ml, 1 mM $CaCl_2$, 20 μM cyt. *c*, 10 mM glucose and a stimulus in a final volume of 2 ml KRP. After a preincubation for 3 min with various concentrations of the priming compound, the reaction was started by adding the stimulus and the absorbance change at 550-540 nm ($\Delta A_{550-540}$) was monitored.

Detection of tyrosyl phosphorylation of neutrophil proteins : Neutrophils (1×10^6 cells) were incubated in 1 ml KRP containing 1 mM $CaCl_2$, 10 mM glucose and 0 - 200 μM of Pro-Pro for 3 min at 37 °C, then 0.5 ml of ice-cold 45 % trichloroacetic acid (final concentration 15 %) containing 1 mM sodium vanadate and 2 mM phenylmethylsulfonylfluoride was added to the reaction mixture. After an incubation for 60 min at 4 °C, the mixture was centrifuged at 10,000 x g for 15 min at 4 °C. The precipitate was washed twice with ice-cold ether/ethanol (1/1) and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [24] with 7.5 % gel. The electrophoresed proteins were transferred onto Imobilon-P membrane (Nippon Millipore Ltd.) using a semidry blotting apparatus (Sartorius) for 35 min at 4.4 mA/cm^2, and the tyrosyl phosphorylated proteins were detected using phosphotyrosine-specific monoclonal antibody (PY-20; ICN Biochemicals, Inc.), peroxidase-conjugated rabbit anti-mouse IgG antibody (E. Y. Laboratories, Inc.), and ECL Western Blotting Detection System (Amersham Japan Co.), as described elsewhere [25]. Molecular weights of the proteins were determined using Prestained Molecular Weight Standards (14,300 - 200,000 molecular weight range; GIBCO BRL).

RESULTS AND DISCUSSION

The patients with prolidase deficiency had large amounts of iminodipeptides, such as Pro-Pro, in the serum [19, 20] and chronic ulcerative dermatitis. In this experiment,

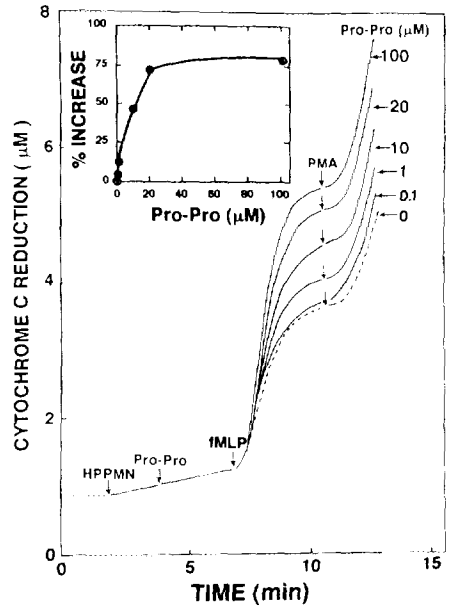

Figure 1. Effect of Pro-Pro on the fMLP-induced O_2^- generation in HPPMN. The neutrophils were preincubated with 0 - 100 μM of Pro-Pro for 3 min at 37 °C prior to the assay of fMLP-induced O_2^- generation. The assays were carried out as described under MATERIALS AND METHODS except that 1 nM PMA was added to the reaction mixture 4 min after the addition of fMLP. The inset shows the relative increase of the O_2^- generation 3 min after the addition of fMLP.

at first, we examined the effect of the various iminodipeptides found in the serum of the patients on the fMLP-dependent increase of O_2^- generation by HPPMN. When the cells were preincubated with Pro-Pro for 3 min, the fMLP-induced O_2^- generation was significantly enhanced in a concentration-dependent manner (Fig. 1). The enhancing effect reached almost plateau level at 20 μM Pro-Pro (Fig. 1, inset) and by 3 min for the preincubation time (data not shown). Furthermore, when PMA was added to the reaction mixture, the O_2^- generation was further increased but the enhancement was independent to the presence of Pro-Pro (Fig. 1 and Fig. 2C). These results indicate that Pro-Pro is a typical priming factor for the agonist-mediated respiration burst of neutrophil.

Table 1 summarizes the contribution of various amino acids and peptides on the fMLP-stimulated O_2^- generation by HPPMN. Among the iminodipeptides containing proline at its C-terminus, not only Pro-Pro but also Gly-Pro and Ala-Pro showed similar stimulative action on the fMLP-induced O_2^- generation, but Ser-Pro, Val-Pro, Leu-Pro and Phe-Pro gave no effect. The iminodipeptides containing hydroxyproline at the C-terminus, Pro-Hyp and Gly-Hyp, did not show stimulative action on the O_2^- generation induced by fMLP. The rates of enhancement by these iminodipeptides follow in the order : Pro-Pro > Gly-Pro > Ala-Pro >> Ser-Pro and Pro-Hyp. On the other hand, the iminodipeptides containing proline at the N-terminus, Pro-Ala and Pro-Gly also showed the stimulative action on the O_2^- generation induced by fMLP. The rates of enhancement

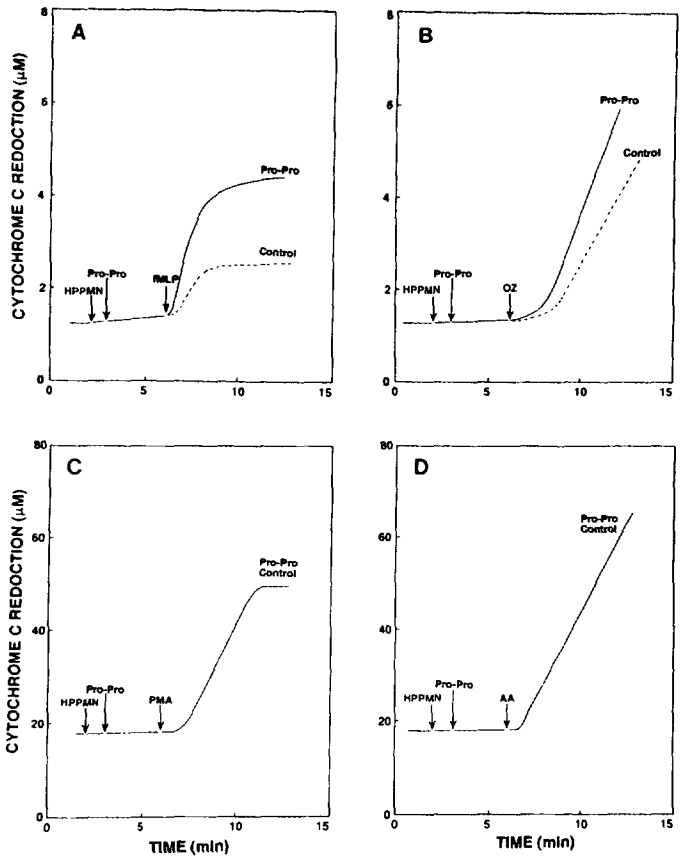

Figure 2. Stimulus-specific effect of Pro-Pro on the O_2^- generation in HPPMN. The neutrophils were preincubated with 100 μM Pro-Pro for 3 min at 37 °C prior to the assay of each stimulus-induced O_2^- generation. The assays were carried out as described under MATERIALS AND METHODS. The final concentration of fMLP (A), OZ (B), PMA (C) and AA (D) added was 12.5 nM, 200 μg/ml, 1 nM and 5 μM, respectively. In the control experiments, the same volume of KRP instead of Pro-Pro was added to the reaction mixture.

by these iminodipeptides follow in the order : Pro-Pro > Pro-Gly > Pro-Ala >> Pro-Hyp. The stimulative action on the O_2^- generation by the iminodipeptides containing proline at the C-terminus, Gly-Pro and Ala-Pro, was higher than those by the counterpart containing proline at the N-terminus, Pro-Gly and Pro-Ala. The iminotripeptides containing proline, Gly-Pro-Ala, Gly-Gly-Pro, Gly-Hyp-Pro, Pro-Gly-Gly and dipeptide without proline, Gly-Gly, and amino acids, Pro and Gly, were also of no effect on the O_2^- generation. These results indicate that only iminodipeptides containing proline at the C-terminus and/or N-terminus, such as Pro-Pro, Gly-Pro, Ala-Pro, Pro-Gly and Pro-Ala were the effective substances on the fMLP-induced O_2^- generation by HPPMN, namely, it was essential to contain proline in the iminodipeptides for the priming action. It might be also noteworthy that Pro-Hyp was not effective and the residue with small side chain, except for proline, was suitable for the partner of proline in the iminodipeptide.

Table 1. Effect of amino acids and peptides on the fMLP-induced O_2^- generation in HPPMN. The neutrophils were preincubated with 100 μM of the priming compound for 3 min at 37 °C prior to the assay of fMLP-induced O_2^- generation. The assays were carried out as described under MATERIALS AND METHODS.

Peptides and Amino acids	% of control
control*	100
Pro	102
Gly	100
Pro-Pro	182
Gly-Pro	168
Ala-Pro	133
Ser-Pro	100
Val-Pro	100
Leu-Pro	100
Phe-Pro	100
Pro-Gly	147
Pro-Ala	122
Pro-Hyp	100
Gly-Hyp	100
Gly-Gly	100
Pro-Gly-Gly	100
Gly-Gly-Pro	100
Gly-Hyp-Pro	100
Gly-Pro-Ala	100

*Without the priming compound.

Effects of Pro-Pro on the O_2^- generation of HPPMN induced by two types of stimuli are shown in Fig. 2. fMLP and OZ were used as the inducer of the receptor-mediated activation of neutrophils. OZ was also used as a phagocytic ligand, since OZ-response is mediated by the receptor of C3b [26]. AA and PMA were used as a membrane perturber and an activator of Ca^{2+}- and phospholipid-dependent protein kinase C [27], respectively. The stimulative action of Pro-Pro similar to that on the fMLP-induced O_2^- generation of HPPMN was observed on the OZ-induced O_2^- generation but not on those induced by PMA or AA. The rates of enhancement by Pro-Pro follow in the order : fMLP > OZ >> PMA and AA, suggesting that the mechanism for priming of HPPMN by Pro-Pro may also involve tyrosine kinase (TK) since the contribution of TK to the priming of HPPMN by tumor necrosis factor has been reported [25].

fMLP-induced phosphorylation of tyrosine residues of HPPMN proteins has been detected by immunoblotting with anti-phosphotyrosine antibody [27, 28]. Using this method, the effect of Pro-Pro on the phosphorylation of tyrosine residues was studied with HPPMN. Non-consistent with previous observations by Gometz-Cambronero *et al.* [28], phosphorylated tyrosines were detected in the HPPMN proteins with molecular weight of 45 , 38 , 32 and 23 kDa (Fig. 3). When incubated with Pro-Pro, phosphorylation of 45 kDa protein markedly increased with time and the phosphorylation depended on the concentration of Pro-Pro. This result is consistent with that reported by Azuma *et al.* [16] who observed the phosphorylation of 42 kDa protein. The

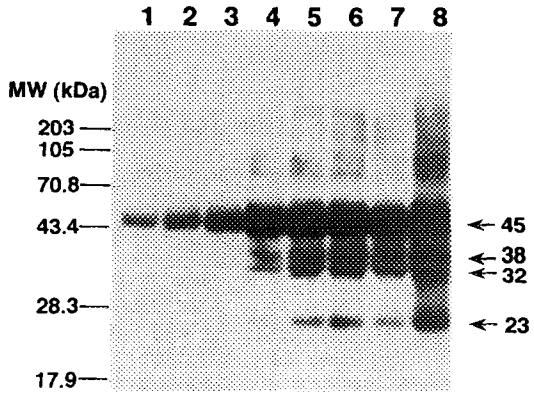

Figure 3. Dose dependent tyrosyl phosphorylation of HPPMN proteins by Pro-Pro. The neutrophils were incubated with 0 - 200 μM of Pro-Pro then the tyrosyl phosphorylated proteins were detected using phosphotyrosine-specific monoclonal antibody (PY-20) as described under MATERIALS AND METHODS. Lane 1, without Pro-Pro ; Lanes 2 - 8, with 1, 5, 10, 20, 50, 100 and 200 μM Pro-Pro, respectively.

phosphorylation of tyrosyl residues of the 45 kDa protein increased parallel with O_2^- generation, and similar enhancement of tyrosyl phosphorylation of the 45 kDa protein was also caused by Gly-Pro (data not shown). These findings suggest that the priming of HPPMN induced by Pro-Pro might be coupled with tyrosyl phosphorylation of cellular proteins, such as the 45 kDa protein. The mechanism of tyrosyl phosphorylation by Pro-Pro is not known yet, but the stimulation of TK by these ligands might occur by some G-protein-dependent mechanism, as reported Akimaru *et al.* [25].

In this study, we found new priming factors, Pro-Pro and several iminodipeptides containing proline, for fMLP-induced O_2^- generation of HPPMN. By using Pro-Pro, the plateau level of the priming effect was given by 20 μM (Fig. 1, inset). This value is about 1/10 of the concentration found in the serum of the patients with prolidase deficiency, 200 μM [20]. This fact suggests that this priming may occur in the peripheral blood of the patient and that PMN of the patient has been primed by the iminodipeptides. Thus, enhancement of tyrosyl phosphorylation of HPPMN by Pro-Pro seems to underlie the triggering mechanism for priming-dependent respiratory burst. The study on the mechanism of O_2^- generation and TK activation in HPPMN primed by Pro-Pro would be the way to clarify the relationships between clinical symptoms and collagen metabolism in patients with prolidase deficiency.

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