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Effect of Cystathionine Ketimine on the Stimulus Coupled Responses of Neutrophils and Their Modulation by Various Protein Kinase Inhibitors

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Human peripheral blood polymorphonuclear leukocytes were preincubated with cystathionine and cystathionine metabolites found in the urine of the patients with cystathioninuria. Among the cystathionine metabolites, cystathionine ketimine significantly enhanced the N-formyl-methionyl-leucyl-phenylalanine-induced superoxide generation, but cystathionine and cyclothionine did not enhance the superoxide generation. Cystathionine ketimine also enhanced superoxide generation induced by opsonized zymosan but not those induced by arachidonic acid and phorbol myristate acetate. Superoxide generation induced by cystathionine ketimine was inhibited by genistein, an inhibitor of tyrosine kinase, and was enhanced by 1-(5-isoquinoline-sulfonyl)-2-methyl-piperazine, an inhibitor of protein kinase C.

Polymorphonuclear leukocytes (PMN) play critical roles in the defense mechanism against microorganisms [1]. When PMN are exposed to various stimuli, one-electron reduction of molecular oxygen by NADPH-oxidase leading to "respiratory burst" is induced [2, 3]. N-formyl-methionyl-leucyl-phenylalanine (fMLP), opsonized zymosan (OZ), arachidonic acid (AA) and phorbol 12-myristate 13-acetate (PMA) 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 polymorphonuclear leukocytes (HPPMN).

Priming and stimulation of PMN have been proposed to occur through different mechanisms [13, 14]. Recently, several reports described that various cytokines and hypotonic condition enhanced the tyrosyl phosphorylation of specific proteins in PMN in the primed stage, suggesting the contribution of tyrosine kinase (TK) to the regulatory mechanism of priming in neutrophils [11, 14–16]. We found that the iminodipeptides such as Pro-Pro enhanced the superoxide (O_2^-) generation induced by fMLP or OZ but not that induced by AA or PMA and that tyrosyl phosphorylation of 45-kDa protein occurred in parallel with the iminodipeptide-dependent enhancement of O_2^- generation in neutrophils [17]. These results also suggest that TK may play critical role(s) in priming and activation of NADPH-oxidase. However, the precise mechanism and the role of tyrosyl phosphorylation in priming and activation of neutrophils are unknown yet.

Cystathioninuria is an autosomal recessive hereditary disorder and the phenotypical homozygotes lead to persistent excretion of large amounts of cystathionine in the urine owing to cysta-

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Abbreviations: PMN, polymorphonuclear leukocytes; fMLP, N-formyl-methionyl-leucyl-phenylalanine; OZ, opsonized zymosan; AA, arachidonic acid; PMA, phorbol 12-myristate 13-acetate; HPPMN, human peripheral blood polymorphonuclear leukocytes; TK, tyrosine kinase; CK, cystathionine ketimine; H-7, 1-(5-isoquinoline-sulfonyl)3-methyl-piperazine; NAc-cysta, N-acetylcystathionine; CMHC, S-(carboxymethyl) homocysteine; HCEHC, S-(2-hydroxy-2-carboxyethyl)homocysteine; β-CEC, S-(2-carboxyethyl)cysteine; HCPC, S(3-hydroxy-3-carboxy-n-propyl)cysteine; OCEHC, S-(2-oxo-2-carboxyethyl)homocysteine; CT, cyclothionine; PKC, Ca^{2+}- and phospholipid-dependent protein kinase.

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thionine γ-lyase deficiency [18]. We previously identified various sulfur-containing amino acids as the cystathionine metabolites in the urine of the patients with cystathioninuria [19–23]. Cystathionine is monodeaminated by a transaminase [24] exhibiting the properties described for glutamine transaminase, and monodeaminated cystathionine mono-oxo-acids cyclize non-enzymatically producing cystathionine ketimine (CK) [25]. CK is reduced enzymatically in mammalian tissues [26–27], and produced cyclothionine (CT) as shown in Fig. 1. Recently, CK was detected in bovine brain cerebellum [26] and human urine [28], but the biochemical and physiological relevance of these sulfur-containing amino acids has not yet been clarified. The presence of these compounds in central nervous system led us to investigate for specific functions.

In the present study, we found that CK, one of the cystathionine metabolites, had a priming effect on O_2^- generation in neutrophils and report herein the results which indicate involvement of TK on NADPH-oxidase activation.

MATERIALS AND METHODS

Chemicals. NADPH, ferricytochrome *c* (cyt. *c*), superoxide dismutase, fMLP, zymosan, AA, PMA and cystathionine were purchased from Sigma Chemical Co. Genistein was from Wako Pure Chemical (Osaka, Japan). 1-(5-isoquinolinesulfonyl)-2-methyl-piperazine (H-7) was from Seikagaku Kogyo Co. (Tokyo, Japan). N-acetyl-cystathionine (NAc-cysta), S-(carboxymethyl)homocysteine (CMHC), S-(2-hydroxy-2-carboxyethyl)homocysteine (HCEHC), S-(2-carboxyethyl)cysteine (β-CEC) and S-(3-hydroxy-3-carboxy-n-propyl)cysteine (HCPC) were prepared as described previously [19, 20]. S-(2-oxo-2-carboxyethyl)homocysteine (OCEHC), cystathionine ketimine (CK) and cyclothionine (CT) were prepared as described by Ricci *et al.* [25]. All other reagents used were of analytical grade and were purchased from Nacalai Tesque Inc. (Osaka, Japan) unless otherwise mentioned.

Isolation of neutrophils. HPPMN were isolated from the peripheral blood of healthy volunteers by Ficoll-Hypaque (Flow Laboratories) density gradient centrifugation [29] and were washed twice with Krebs-Ringer-Phosphate solution (KRP; pH 7.4) [30]. The cells were counted and resuspended in KRP at concentration of 1×10^8 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]. The standard assay mixture consisted of 1×10^6 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 (cystathionine or the metabolites, 0–100 μM), the reaction was started by adding the stimulus and the absorbance change at 550–540nm ($\delta A_{550-540}$) was monitored. 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 [31].

RESULTS AND DISCUSSION

Large amounts of cystathionine (1236.08 mg/g creatinine) and the metabolites are excreted in the urine of a patient with cystathioninuria [19–23]. The concentrations of CT and CK in the urine of this patient were 318.56 and 3.61 mg/g creatinine, respectively. The patient excreted 20 times more CK in the urine than normal person did [23]. In the present study, we examined the effect of these sulfur-containing amino acids on a O_2^- generation by HPPMN. Incubation of the cells with cystathionine or the metabolites at 30 μM did not induced the O_2^- generation. However, when the cells were preincubated with the sulfur-containing amino acids for 3 min, enhancement of an fMLP-induced O_2^- generation was observed. In the sulfur-containing amino acids examined, CK,

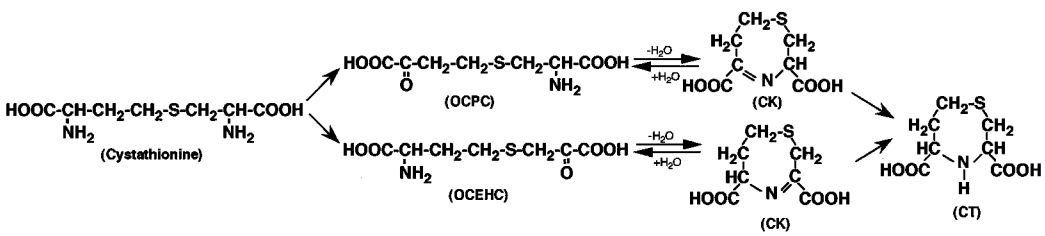

FIG. 1. Unusual metabolism of cystathionine in a patient with cystathioninuria.

TABLE 1
Effect of Cystathionine and Cystathionine Metabolites on the fMLP-Induced O_2^- generation in HPPMN

Sulfur amino acids (30 μM)	% of control mean ± SD[a]
Control[b]	100.0 ± 0.00
NAc-Cysta	102.1 ± 1.86
Cysta	98.5 ± 1.33
CK	274.3 ± 11.91
CT	99.3 ± 1.32
CMHC	99.3 ± 4.68
HCEHC	144.4 ± 3.50
β-CEC	99.9 ± 4.69
HCPC	116.8 ± 9.95

The neutrophils were preincubated with 30 μM priming compound for 3 min at 37°C prior to the assay of fMLP-induced O_2^- generation.
[a] $n = 3$.
[b] Without sulfur amino acid.

HCEHC and HCPC showed a stimulative effect on the fMLP-induced O_2^- generation by HPPMN while cystathionine and other cystathionine metabolites gave no effect (Table 1). The rates of enhancement by the effective sulfur-containing amino acids followed in the order: CK >> HCEHC > HCPC (at 30 μM). Especially, the stimulative effect of CK was remarkable and the fMLP-induced O_2^- generation was enhanced by CK in a concentration-dependent manner (Figs. 2 and 3A). The stimulative effect of NAc-cysta was negrigible at 30 μM but was significant at 100 μM. On the contrary, the stimulative effect on the O_2^- generation of HCEHC and HCPC was not observed at 100μM (Fig. 2). Cystathionine and CT gave no significant effect even at high concentration, 300 μM (data not shown).

Figure 3 shows the effect of CK on the O_2^- generation induced by two types of stimulus. 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 [32]. AA and PMA were used as a membrane perturber and an activator of Ca^{2+}- and phospholipid-dependent protein kinase C (PKC) [33], respectively. CK enhanced not only the fMLP-induced O_2^- generation but also the OZ-induced O_2^- generation, while the stimulative effect was not observed on the O_2^-

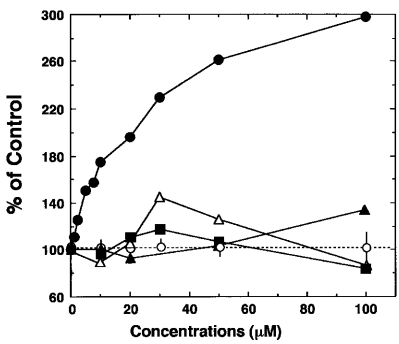

FIG. 2. Effect of cystathionine and the metabolites on fMLP-induced O_2^- generation by HPPMN. The cells were preincubated with cystathionine and the metabolites (0–100 μM) for 3 min prior to the addition of fMLP. The experimental conditions were described under MATERIALS AND METHODS. ●, CK; △, HCEHC; ▲, NAc-cysta; ■, HCPC; ○, Cysta CT, CMHC and β-CEC follow the line by Cysta.

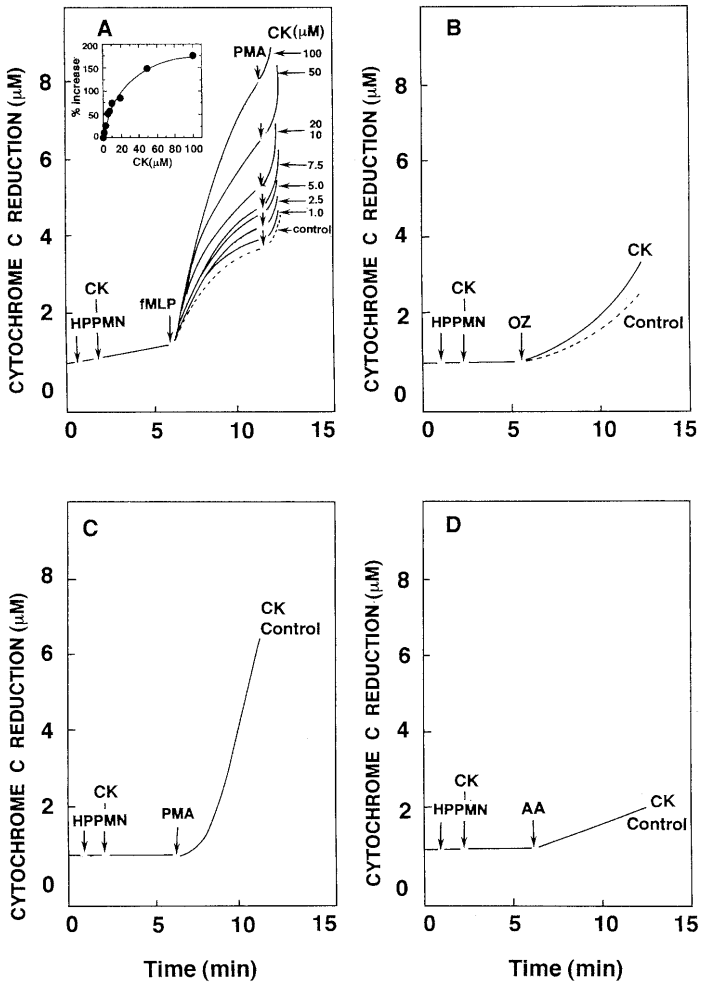

FIG. 3. Stimulus-specific effect of CK on the O_2^- generation in HPPMN. The cells were preincubated with CK for 3 min at 37 °C prior to the assay of each stimulus-induced O_2^- generation: A, 0–100 μM; B-D, 30 μM. The assays were carried out as described under MATERIALS AND METHODS except that 1 nM PMA was added to the reaction mixture at 4 min after the addition of fMLP (A). The final concentrations of fMLP (A), OZ (B), PMA (C) and AA (D) added were 12.5 nM, 200 μg/ml, 5 μM and 1 nM, respectively. In the control experiment, the same volume of KRP instead of CK was added to the reaction mixture. The inset in A shows the relative increase of the O_2^- generation at 3 min after the addition of fMLP.

generation induced by PMA or AA. When PMA was added to the reaction mixture for fMLP-induced O_2^- generation, the O_2^- generation was further increased but the enhancement was independent to the presence of CK (Figs. 3A and 3C). The rates of enhancement by CK followed in the order: fMLP > OZ >> PMA and AA. These results indicate that CK is a typical priming factor for the agonist-mediated respiration burst of the neutrophils.

In our previous study, we obtained the results which suggest participation of TK in the mechanism for priming of HPPMN by Pro-Pro [17], and contribution of TK to the priming of HPPMN by tumor necrosis factor was also reported [34]. To determine whether PKC and TK participate in the mechanism for priming of HPPMN, the effect of protein kinase inhibitors on the fMLP-induced O_2^- generation was examined. As shown in Fig. 4, the fMLP-induced O_2^- generation by CK-treated HPPMN was inhibited by genistein, an inhibitor of TK, in a concentration-dependent

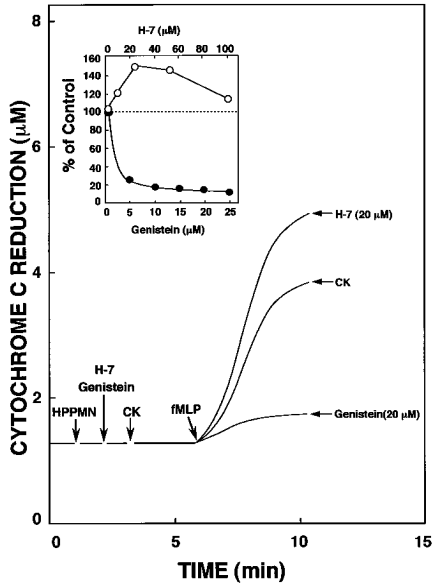

FIG. 4. Effect of protein kinase inhibitors on fMLP-induced O_2^- generation by CK-treated HPPMN. The cells were preincubated with 30 μM CK in the presence or absence of 20 μM of a protein kinase inhibitor prior to addition of fMLP. Other conditions were as described under MATERIALS AND METHODS. The inset shows the relative amount of the O_2^- generation at 3 min after the addition of fMLP in the presence of the protein kinase inhibitor. ●, genistein; ○ H-7.

manner whereas H-7, an inhibitor of PKC, rather enhanced the O_2^- generation. The similar results were obtained by using other inhibitors, herbimycin A for TK and staurosporine for PKC (data not shown).

In the present study, we found a new priming factor, CK, for fMLP-induced O_2^- generation by HPPMN and obtained the results suggesting that TK participates in the fMLP-mediated O_2^- generation by CK-treated HPPMN. CK was detected in bovine brain cerebellum, and the study on the binding of CK to bovine brain cortex membranes is on going. Fontana *et al.* [35] found that CK bound to brain membranes with high-affinity, and CT, a reduced product of CK, was completely ineffective in competing with the binding of CK. CK binds not only to brain membranes but also to the membranes prepared from bovine heart and kidney. Studies on the mechanisms of O_2^- generation and TK activation in HPPMN primed by CK may make it possible to clarify the physiological role of CK in patients with cystathioninuria.

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