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Effect of cystathionine as a scavenger of superoxide generated from human leukocytes or derived from xanthine oxidase in vitro

Kouichirou Wada *, Yoshinori Kamisaki, Kentaro Nakamoto, Tadao Itoh

Department of Clinical Pharmacology, Faculty of Medicine, Tottori University, 86 Nishimachi, Yonago 683, Japan

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Abstract

We studied the direct effects of cystathionine on human leukocyte-generated or xanthine-xanthine oxidase-derived superoxide radicals in vitro. Washed leukocyte suspensions (10^6 cells/ml) prepared from healthy male volunteers were stimulated with phorbol myristate acetate (1 μM) or opsonized zymosan (1 mg/ml) to generate superoxide radicals, which were measured with a 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-α]pyrazin-3-one hydrochloride (MCLA)-chemiluminescence method. Cystathionine (30 μM to 10 mM) significantly reduced superoxide radical-dependent chemiluminescence in the leukocyte system in a concentration-dependent manner. In addition, in the two different methods of determination of superoxide radicals (MCLA chemiluminescence and nitroblue tetrazolium reduction), cystathionine significantly scavenged the superoxide radicals derived from the xanthine-xanthine oxidase system. However, cystathionine did not inhibit the activity of xanthine oxidase during superoxide generation. On the other hand, cystathionine did not show a scavenging effect against hydroxyl radicals derived from Fe^{2+}-H_2O_2 on the erythrocyte membrane. These results indicate that cystathionine itself may possess a scavenging function against superoxide radicals rather than against hydroxyl radicals in vitro.

Keywords: Superoxide radical; Leukocyte, human; Cystathionine

1. Introduction

Reactive oxygen species are known to be associated with pathological conditions, e.g. inflammation, ischemia-reperfusion injury and atherosclerosis, in many diseases (Davies, 1993; Hall, 1993; Niki et al., 1993; Schapira et al., 1993). In particular, superoxide radicals have been proposed to play important roles in cell injury, directly or indirectly. Therefore, it is necessary to scavenge the excessively produced superoxide radicals rapidly to protect from cell injury. Superoxide dismutase and low molecular weight substances such as ascorbic acid or α-tocopherol are considered to be main scavengers of superoxide radicals (Freisleben and Packer, 1993). Cysteine, glutathione and other sulfhydryl donors also have a radical-scavenging action, and accordingly, may act as anti-oxidants in vivo (Asada and Kanematsu, 1976; Niki et al., 1985; Devasagayam et al., 1991; Meister, 1994). Glutathione especially is thought to be a most important reductive agent involved in the glutathione peroxidase system (Ross et al., 1985; Meister, 1994; Winterbourn and Metodiewa, 1994). Thus, many studies connected with the in vivo anti-oxidant activity of sulfhydryl group-containing small molecules have been reported. On the other hand, little work has been reported on the anti-oxidant activity of sulfur-containing molecules, such as cystathionine or methionine.

Cystathionine, a metabolic precursor of cysteine, is converted to cysteine by γ-cystathionase under physiological conditions in liver. Thus, in fact, cystathionine has been reported to cause hepatoprotective effects against acetaminophen-induced necrosis (Kitamura et al., 1989). However, direct effects of cystathionine against reactive oxygen species such as superoxide radicals have not been examined. Therefore we studied the in vitro scavenging effects of cystathionine on superoxide radicals which were generated from stimulated

* Corresponding author. Tel.: (81) 859-34-8014, fax: (81) 859-34-8140.

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human leukocytes or derived from the xanthine-xanthine oxidase system. Further, we compared these effects with those on hydroxyl radicals.

2. Materials and methods

2.1. Reagents

The luminescence reagent, 2-methyl-6-[*p*-methoxyphenyl]-3,7-dihydroimidazo[1,2-α]pyrazin-3-one hydrochloride (MCLA) was obtained from Tokyo Kasei Co. (Tokyo, Japan). MCLA was prepared as previously described (Nishida et al., 1989; Saniabadi and Nakano, 1993) to detect superoxide radicals.

Cystathionine, phorbol 12-myristate 13-acetate, zymosan A and superoxide dismutase (from human erythrocyte) were obtained from Sigma (St. Louis, USA). Phorbol myristate acetate was dissolved in dimethyl sulfoxide and was diluted with physiological saline to a final concentration of 1 μM. The final concentration of dimethyl sulfoxide used in the present experiments was 0.05% or less.

Superoxide dismutase (specific activity of 3610 units/mg protein) was dissolved in saline and stored at −30°C for daily use. Final concentrations of superoxide dismutase were 1–10 units/ml. Zymosan A was opsonized according to Saniabadi and Nakano (1993). Xanthine oxidase from buttermilk was obtained from Wako Chemicals (Tokyo, Japan). All chemicals were of reagent grade.

2.2. Measurement of superoxide radicals generated from leukocytes or the xanthine-xanthine oxidase system using the MCLA-chemiluminescence method

Leukocyte suspensions were prepared from healthy volunteers by the modified method of Saniabadi and Nakano (1993). Briefly, venous blood was collected into plastic tubes containing heparin (50 units/ml) and 6% dextran-T70 (blood : dextran, 9 : 1 v/v). After gentle mixing, the blood was left undisturbed for 60–70 min. The plasma phase containing the leukocytes, platelets and residual erythrocytes was harvested and centrifuged at $200 \times g$ for 10 min to remove the platelets. Then, the pellet was resuspended in 0.168 M NH_4Cl for lysis of erythrocytes and centrifuged at $250 \times g$ for 10 min. After twice washing with saline, the pellet was resuspended in Hanks' balanced salt solution containing 1.26 mM of Ca^{2+} and 0.9 mM of Mg^{2+}, pH 7.4. Leukocyte viability was 98–100% (Trypan blue exclusion test). For these experiments, the leukocyte count was adjusted to 10^6 cells/ml.

Human leukocyte-generated superoxide radical was measured by the modified method of Saniabadi and Nakano (1993). Briefly, leukocyte suspension (1.5 ml) was incubated in the reaction vial for 1 min to equilibrate to 37°C and 1 μM of MCLA was then added. Stimulation was initiated by addition of phorbol myristate acetate (1 μM) or opsonized zymosan (1 mg/ml). Chemiluminescence intensity was measured with a Hitachi F-2000 fluorometric reader (Japan). Total chemiluminescence intensity was measured for 5 min.

Superoxide generation from xanthine-xanthine oxidase system was done with the modified method of Beauchamp and Fridovich (1971). Briefly 1.5 ml of reaction medium (Tris-HCl buffer, pH 7.4) containing 10 U/ml of xanthine oxidase was incubated in the reaction vial for 1 min, and 1 μM of MCLA was added. Xanthine (30 μM) was added to generate superoxide radicals. Total chemiluminescence intensity was measured for 5 min.

2.3. Measurement of superoxide radicals derived from xanthine-xanthine oxidase system using the nitroblue tetrazolium method

Measurement of superoxide radicals derived from the xanthine-xanthine oxidase system using the nitroblue tetrazolium method was done with the modified method of Nishikimi et al. (1972). Briefly 1.5 ml of reaction medium (Tris-HCl buffer, pH 7.4) containing 10 U/ml of xanthine oxidase and 250 μM of nitroblue tetrazolium in the reaction vial, and cystathionine or superoxide dismutase was added. Xanthine (100 μM) was added to generate superoxide radicals for 5 min at 37°C. The superoxide radicals generated were estimated by spectrophotometric measurement (560 nm) of the product of the reduction of nitroblue tetrazolium. Superoxide radical generation was measured as increased absorbance of 560 nm for 5 min.

2.4. Measurement of uric acid in the xanthine-xanthine oxidase system

To rule out a possible inhibition of xanthine oxidase by cystathionine, we measured the concentration of uric acid generated during superoxide generation in the xanthine-xanthine oxidase system. Briefly 1.5 ml of reaction medium (Tris-HCl buffer, pH 7.4) containing 10 U/ml of xanthine oxidase in the reaction vial, and cystathionine (final concentration, 3 mM) or vehicle was added. Xanthine (100 μM) was added to generate superoxide radicals for 5 min at 37°C. After the incubation, 50 μl of reaction medium was used for the measurement of uric acid, using a uric acid determination kit (Uric acid C-test wako, Wako Chemicals, Tokyo, Japan).

2.5. Determination of lipid peroxidation of erythrocyte membrane ghosts by hydroxyl radicals

Human venous blood from healthy volunteers was collected in heparin-containing tubes. Erythrocytes

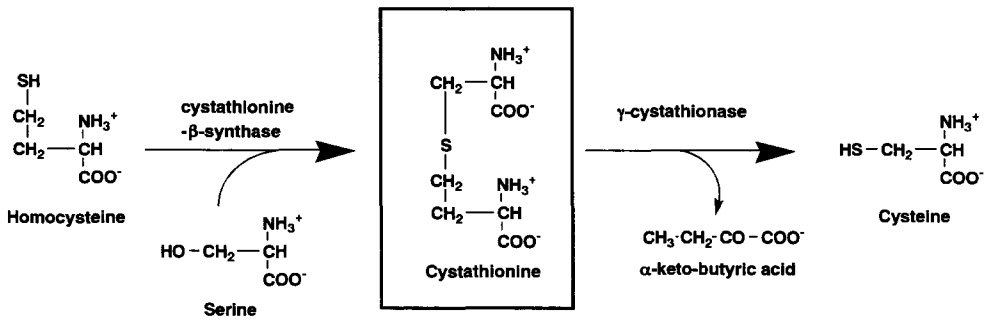

Fig. 1. Structure and metabolism of cystathionine in vivo.

were pelleted by centrifugation and washed in saline. White ghosts were prepared by repeated washing and lysis in 5 mM phosphate buffer (Steck and Kant, 1974; Sills et al., 1994). Erythrocyte membrane ghosts were diluted with saline to a final concentration of 0.9–1.1 mg protein/ml and used as targets of lipid peroxidation.

Hydrogen peroxide (3 mM) and $FeSO_4$ (5 mM) were then added immediately to erythrocyte membrane ghost suspensions (1 ml) with or without radical scavengers. Ghost suspensions were incubated at 37°C for 30 min (Sills et al., 1994). Hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghosts was determined by measuring thiobarbituric acid reactive substances according to methods described previously (Buege and Aust, 1978; Svingen et al., 1979).

2.6. Statistics

All results are expressed as means ± S.E.M. Statistical comparisons were done with Student's *t*-test or Scheffe's multiple comparison test. Results were considered significantly different when $P < 0.05$.

3. Results

3.1. Metabolism of cystathionine

Cystathionine is converted to cysteine by γ-cystathionase under physiological conditions in the liver (Fig. 1). However, the conversion to cysteine was not observed in our in vitro experiments, because no free SH group could be determined by the method of Ellman (1959) under the present experimental conditions (data not shown).

3.2. Effect of cystathionine on leukocyte-generated superoxide radicals

Fig. 2 shows the comparison of the scavenging effects on human leukocyte-generated superoxide radicals stimulated with phorbol myristate acetate (1 μM) of cystathionine and other radical scavengers. The

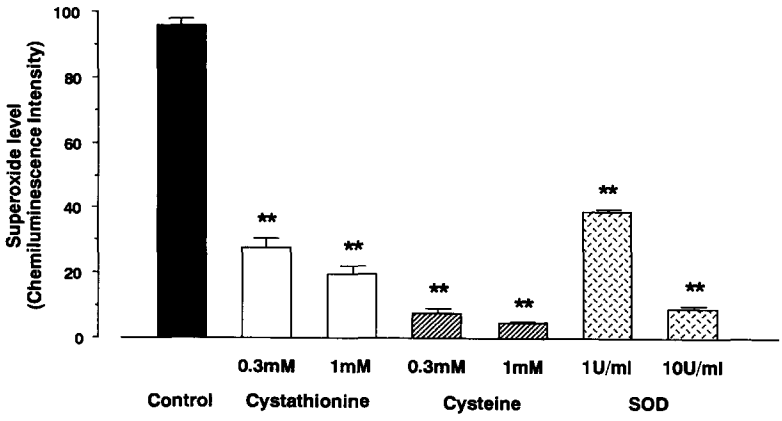

Fig. 2. Comparison of scavenging effects of cystathionine and other radical scavengers on human leukocyte-generated superoxide radicals stimulated by phorbol myristate acetate (1 μM). $^{**}P < 0.01$ each treatment vs. control. Each column represents the mean ± S.E.M. derived from six to eight observations.

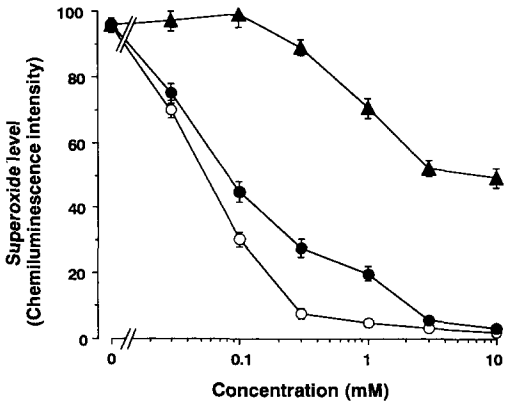

Fig. 3. Concentration-dependent scavenging effect of cystathionine on human leukocyte-generated superoxide radicals stimulated by phorbol myristate acetate (1 μM). Closed circles (●) represent cystathionine, open circles (○), cysteine and closed triangles (▲), methionine. Superoxide levels were determined from the chemiluminescence intensity of MCLA. Each point represents the mean ± S.E.M. derived from six to eight observations.

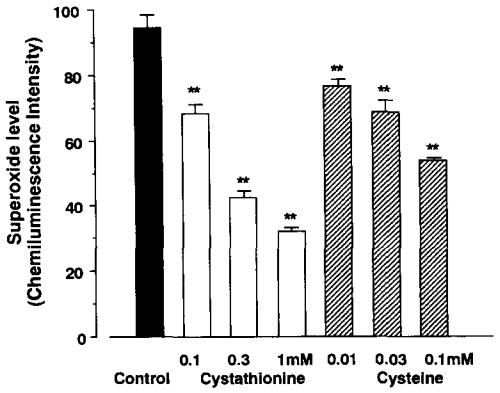

Fig. 4. The scavenging effects of cystathionine and cysteine on xanthine-xanthine oxidase-derived superoxide radicals in the MCLA-chemiluminescence method. Superoxide levels were determined from the chemiluminescence intensity of MCLA. $^{**}P < 0.01$ each treatment vs. control. Each column represents the mean ± S.E.M. derived from six to eight observations.

scavenging effects of cystathionine (0.3 mM and 1 mM) were between those of 1 U/ml and 10 U/ml of superoxide dismutase, a typical superoxide scavenger. The scavenging effect of cystathionine on superoxide radicals was concentration-dependent (Fig. 3). Cysteine, a typical radical scavenger, also showed the scavenging effect in a concentration-dependent manner. The efficacy ratio of cystathionine to cysteine was approximately 1/3. On the other hand, methionine, which is a sulfur-containing amino acid, and may react with superoxide radicals (Griffith, 1987; Johnson and Westrick, 1987; Scislowski and Davis, 1987; Maier et al., 1991) showed a weak scavenging effect. The same results were observed when opsonized zymosan (1 mg/ml) was used as a leukocyte stimulant (data not shown).

3.3. Effect of cystathionine on xanthine-xanthine oxidase-derived superoxide radicals in the MCLA-chemiluminescence method

Fig. 4 shows the effects of cystathionine and cysteine on xanthine-xanthine oxidase-derived superoxide radicals in the MCLA-chemiluminescence method. Cystathionine also showed the scavenging effect on xanthine-xanthine oxidase-derived superoxide radicals. These results indicate that cystathionine itself is able to scavenge the superoxide radicals in vitro, since the xanthine-xanthine oxidase system does not involve the enzymes which metabolize cystathionine.

3.4. Effect of cystathionine on xanthine-xanthine oxidase-derived superoxide radicals in the nitroblue tetrazolium reduction method

Fig. 5 shows the effects of cystathionine and superoxide dismutase on xanthine-xanthine oxidase-derived superoxide radicals in the nitroblue tetrazolium reduction method. Cystathionine also showed the scavenging effect on xanthine-xanthine oxidase-derived superoxide radicals in this method. The same results for the scavenging effect of cystathionine on the xanthine-xanthine oxidase-derived superoxide radicals were obtained from two completely different methods for determination of superoxide radical.

3.5. Measurement of uric acid in the xanthine-xanthine oxidase system

In the xanthine-xanthine oxidase system, the control generated 107.6 ± 3.5 μM (mean ± S.E.M., $n = 6$) of

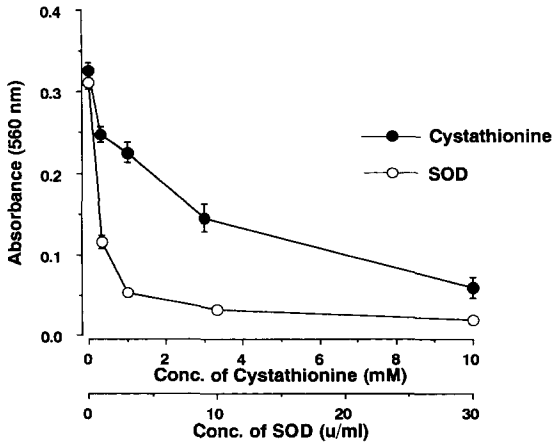

Fig. 5. The concentration-dependent scavenging effects of cystathionine and superoxide dismutase (SOD) on xanthine-xanthine oxidase-derived superoxide radicals in the nitroblue tetrazolium reduction method. Closed circles (●) represent cystathionine and open circles (○), SOD. Superoxide levels are indicated as increased absorbance at 560 nm for 5 min.

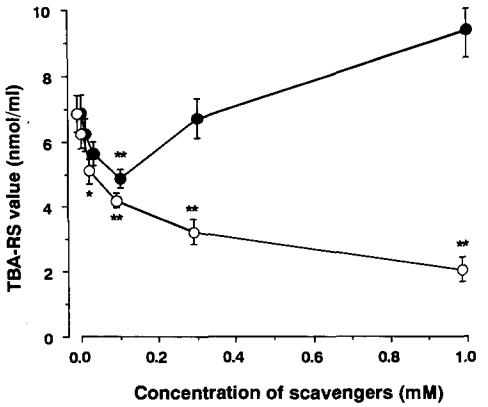

Fig. 6. Effect of cystathionine and cysteine on ·OH-derived lipid peroxidation of erythrocyte membrane ghosts. Closed circles (●) represent cystathionine and open circles (○), cysteine. Lipid peroxidation was determined from thiobarbituric acid-reactive substances (TBA-RS) values (means ± S.E.M). $^{**}P < 0.01$ each treatment vs. control (with no scavengers) derived from six to eight observations.

uric acid during superoxide generation. The presence of cystathionine (3 mM) did not inhibit the generation of uric acid (102.8 ± 12.2 μM, mean ± S.E.M., $n = 6$), although this concentration of cystathionine reduced the superoxide level. These results indicate that cystathionine itself scavenges the superoxide radicals without inhibiting the activity of xanthine-xanthine oxidase in vitro.

3.6. Effect of cystathionine on hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghosts

The effect of cystathionine on hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghosts is shown in Fig. 6. Cystathionine reduced the lipid peroxidation (thiobarbituric acid-reactive substances) level of erythrocyte membrane ghosts at the concentration of 30 to 100 μM. However, more than 300 μM of cystathionine did not reduce the lipid peroxidation level. The effect of cystathionine on hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghosts showed two phases. Cysteine, which is also reported to scavenge hydroxyl radicals (Asada and Kanematsu, 1976; Niki et al., 1985; Devasagayam et al., 1991), significantly reduced the lipid peroxidation level in a concentration-dependent manner.

4. Discussion

Some diseases concerned with radicals involve the participation of sulfur-containing amino acids (Heinecke et al., 1987; Maier et al., 1991). Further, a role of cystathionine as the storage pool of thiols in organs has been reported (Rusakow et al., 1993). Therefore, cystathionine, which is more stable than cysteine in vivo, may be useful as a radical scavenger and source of thiols in the living body.

In this study, we have shown the scavenging effects of cystathionine on human leukocyte-generated or xanthine-xanthine oxidase-derived superoxide radicals in vitro. Cystathionine is converted to cysteine by γ-cystathionase under physiological conditions in the liver. The existence of a metabolic system of cystathionine in leukocytes is not yet clear. However, the enzymic conversion of cystathionine to cysteine in in vitro experiments of our study may not have occurred because detection of the SH group revealed that the conversion was not actually observed. These results suggest that cystathionine may act directly as a scavenger of superoxide radicals without conversion to cysteine in vitro.

Superoxide radicals are mainly generated from several different enzymic systems in the body. The NADPH-oxidase system in leukocytes (Jones et al., 1993) and the xanthine oxidase system (Beauchamp and Fridovich, 1971) were used in this study. Cystathionine significantly decreased the superoxide levels of both the NADPH-oxidase system of leukocytes and the xanthine oxidase system in a concentration-dependent manner. These two systems are completely different from each other as to the mechanism of generation of superoxide radicals. No evidence has been reported for the inhibition by cystathionine of these two enzymes. However, in the determination of uric acid during superoxide generation, cystathionine did not inhibit the activity of xanthine-xanthine oxidase. Further, in the preliminary experiment, we also observed the quenching effect on the subsequent addition of cystathionine on the superoxide radical. These results suggest that the reduction of superoxide radicals may be due to the direct scavenging action of cystathionine, but not to the inhibition of enzymes.

In the present study, we used two different methods, MCLA-chemiluminescence and nitroblue tetrazolium reduction, to detect superoxide radicals derived from the xanthine-xanthine oxidase system. The nitroblue tetrazolium reduction method is widely used to measure superoxide generated in vitro (Nishikimi et al., 1972). However, the MCLA-chemiluminescence method is reported to be superior to the nitroblue tetrazolium reduction method as to specificity for detection of superoxide radicals, because nitroblue tetrazolium is easily reduced by the contaminated reducing substances (Beauchamp and Fridovich, 1971; Nishikimi et al., 1972; Nishida et al., 1989; Saniabadi and Nakano, 1993). In spite of its low specificity, we used the nitroblue tetrazolium reduction method to rule out the possibility that cystathionine interferes with the MCLA-chemiluminescence assay. We obtained similar results for the scavenging effect of cystathionine on the

xanthine-xanthine oxidase-derived superoxide radicals from these two completely different methods for superoxide radical determination. Therefore, an interaction between cystathionine and MCLA can be ruled out.

The scavenging mechanism of cystathionine on superoxide radical was not clarified in this study. However, it is reported that free methionine or methionine residues in proteins are attacked and oxidized by the radical (Johnson and Westrick, 1987; Scislowski and Davis, 1987; Maier et al., 1991). Methionine or methionine residues in proteins were oxidized to methionine sulfoxide and consequently to methionine sulfone (Johnson and Westrick, 1987; Griffith, 1987). Together with the results for the mechanism of methionine action, our observations suggest that the sulfur group of cystathionine may react with the superoxide radical to form sulfoxide and sulfone, although further studies are needed to confirm the existence of this mechanism.

The scavenging effect of cystathionine on hydroxyl radical-induced lipid peroxidation of erythrocyte membrane ghost was not strong, although cysteine significantly reduced the lipid peroxidation level in a concentration-dependent manner. The scavenging effect of cystathionine against hydroxyl radicals seems to have an optimal concentration because of its two-phase effect, although further studies are needed to confirm this. These results indicate that the in vitro scavenging action of cystathionine may prefer superoxide radicals to hydroxyl radicals.

In conclusion, the present data suggested the possibility that cystathionine itself may play an important role as a superoxide radical scavenger.

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