ELSEVIER

European Journal of Pharmacology 449 (2002) 183–189

www.elsevier.com/locate/ejphar

S-carboxymethylcysteine inhibits neutrophil activation mediated by *N*-formyl-methionyl-leucyl-phenylalanine

Yukio Ishii *, Toru Kimura, Yuko Morishima, Mie Mochizuki, Akihiro Nomura, Tohru Sakamoto, Yoshiyuki Uchida, Kiyohisa Sekizawa

Department of Respiratory Medicine, Institute of Clinical Medicine, University of Tsukuba, 1-1-1 Ten-nohdai, Tsukuba, Ibaraki 305-0006, Japan

Received 26 April 2002; accepted 18 June 2002

Abstract

In this study, the possible mechanisms of action for the inhibitory effects of *S*-carboxymethylcysteine on the activation of human neutrophils by *N*-formyl-methionyl-leucyl-phenylalanine (FMLP) were investigated. Preincubation of neutrophils with more than 10 μg/ml of *S*-carboxymethylcysteine was found to impair neutrophil chemotactic activity toward FMLP, and to inhibit FMLP-mediated neutrophil adherence to pulmonary vascular endothelial cells. Preincubation of neutrophils with 10 and 100 μg/ml of *S*-carboxymethylcysteine decreased in the production of inositol 1,4,5-triphosphate (IP_3) and diacylglycerol in neutrophils stimulated with FMLP, respectively. Preincubation of neutrophils with *S*-carboxymethylcysteine did not affect the cellular cyclic AMP (cAMP) levels in neutrophils stimulated with FMLP. *S*-carboxymethylcysteine inhibited the enzymatic activity of phosphatidyl inositol-specific phospholipase C in vitro in a concentration-dependent manner. These findings indicate that *S*-carboxymethylcysteine attenuates FMLP-stimulated neutrophil activation at least in part by inhibiting phosphatidyl inositol-specific phospholipase C-mediated signal transduction.

Keywords: *S*-carboxymethylcysteine; Neutrophil; FMLP (*N*-formyl-methionyl-leucyl-phenylalanine); Phospholipase C; Phosphatidyl inositol-specific

1. Introduction

Neutrophils are known to be a major cellular component of host defense mechanisms (Gallin, 1988; Smith, 1994). Neutrophils activated at the tissue site, however, can cause tissue injury by releasing their toxic content, including oxygen metabolites, proteolytic enzymes, and cationic proteins, and by cleaving matrix proteins into chemotactic factors that can exacerbate inflammation by attracting additional inflammatory cells (Dallegri and Ottonello, 1997; Weiss, 1989; Vartio et al., 1981).

Neutrophils can be activated by several mediators, including formylated chemotactic peptides such as *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), complement C5a fragment, and leukotriene B_4 (Palmblad, 1984). These chemotactic factors can activate neutrophils to enhance chemotaxis, adherence to endothelial cells, and responses to cytokines via their specific cell-surface receptors for the chemotactic agents (Lew, 1990).

S-carboxymethylcysteine (Fig. 1) is a mucolytic drug and has been used in the treatment of different respiratory diseases characterized by abnormal mucus secretion. In addition to its mucolytic effects, it has been recently demonstrated that *S*-carboxymethylcysteine inhibits neutrophil infiltration into the lung during lung injury induced by bleomycin (Hirata and Ohashi, 1995). *S*-carboxymethylcysteine also inhibits neutrophil chemotactic activity toward FMLP, zymosan-activated serum, and leukotriene B_4 in vitro (Hirata and Ohashi, 1995).

In the present study, therefore, we investigated the cellular mechanisms of *S*-carboxymethylcysteine's inhibitory effects on neutrophil activation mediated by FMLP. Investigations of signal transduction mechanisms of FMLP receptors have demonstrated that the binding of FMLP to its receptors on the surface of neutrophils leads to activation of heterotrimeric G-proteins, stimulation of phosphatidyl inositol-specific phospholipase C, and subsequently to inflammatory responses (Di Virgilio et al.,

* Corresponding author. Tel./fax: +81-298-53-3144.
E-mail address: ishii-y@md.tsukuba.ac.jp (Y. Ishii).

0014-2999/02/$ - see front matter © 2002 Elsevier Science B.V. All rights reserved.
PII: S0014-2999(02)01981-7

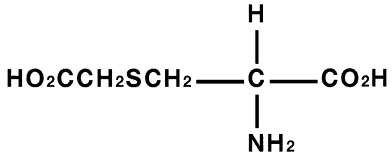

Fig. 1. Chemical structure of *S*-carboxymethylcysteine.

1985; Verghese et al., 1986; Dillon et al., 1987; Amatrude et al., 1995).

2. Materials and methods

2.1. Preparation of human neutrophils

Blood from normal human volunteers was drawn by venopuncture into a 0.1 volume of 3.8% citrate in conical plastic tubes. Neutrophils were purified by standard techniques of dextran sedimentation, centrifugation on Ficoll-Hypaque (Pharmacia, Piscataway, NJ) and hypotonic lysis of erythrocytes. The cell suspensions contained a minimum of 95% neutrophils, and cell viability always exceeded 98%, as determined by trypan blue exclusion.

2.2. Neutrophil chemotactic assay

Neutrophils were incubated with various concentrations of *S*-carboxymethylcysteine or U73122 (Calbiochem-Novabiochem, San Diego, CA) at 37 °C for 1 h. *S*-carboxymethylcysteine was kindly provided by Kyorin Pharmaceutical (Tokyo, Japan). Some of the neutrophils were co-incubated with 1,2-bis-(*o*-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, tetraacetoxymethyl ester (BAPTA-AM; Calbiochem-Novabiochem) to chelate intracellular Ca^{2+}. Neutrophils were washed twice in Hank's balanced salt solution (GIBCO, Grand Island, NY) and then resuspended to a concentration of 1.5×10^6/ml in Hank's balanced salt solution containing 2% bovine serum albumin. The migration assay was performed with 50 μl of neutrophil suspension in the upper wells of a 48-well modified Boyden chamber (Neuro Probe, Cabin John, MD) equipped with a Nucleopore polycarbonate filter (3-μm pore size). The lower wells contained 25 μl of Hank's balanced salt solution containing 2% bovine serum albumin and 10^{-6} M FMLP. Following chamber incubation for 40 min at 37 °C, the filter was stained with Diff-Quik (American Scientific Products, McGraw Park, IL), and the neutrophils reaching the lower surface were counted in 10 oil-immersion fields (× 100) for each duplicate set of wells. In each set, the migration numbers of neutrophils untreated with *S*-carboxymethylcysteine were used as a positive control.

2.3. Cell-adhesion assay

Human pulmonary vascular endothelial cells (Sanko Junyaku, Tokyo, Japan) were plated in 48-well plates and incubated in culture medium until confluence. The cells were then treated with 15 U/ml of interleukin-1β (Sigma), and 25 ng/ml of tumor necrosis factor-α (Sigma) in RPMI 1640 (GIBCO) containing 1% fetal bovine serum for 3 h at 37 °C in a humidified atmosphere of 5% carbon dioxide.

Neutrophils were treated simultaneously with various concentrations of *S*-carboxymethylcysteine or U73122 and labeled with sodium chromate ^{51}Cr (18.5×10^7 Bq/ml; New England Nuclear, Boston, MA) at 37 °C for 1 h. Some were co-incubated with BAPTA-AM. Neutrophils were then washed twice with PBS and then resuspended in RPMI1640 containing 1% fetal bovine serum at a concentration of 2×10^6/ml. The labeling efficacy was not affected by the *S*-carboxymethylcysteine concentrations.

After a 3-h incubation of the endothelial cells with interleukin-1β and tumor necrosis factor-α, cells were washed twice with RPMI1640. Radiolabeled neutrophils were added to each well at a density of 5×10^5. Plates were incubated at 37 °C for 30 min in a humidified atmosphere with 5% carbon dioxide. After the incubation, the medium and the nonadherent neutrophils were carefully aspirated and washed twice. The remaining pellet was then solubilized with 1 N sodium hydroxide, and the radioactivity in the lysate was measured. The adherence of each well is expressed as Adherence (%) = ^{51}Cr (cpm) in pellet/total ^{51}Cr (cpm) × 100.

2.4. Measurement of inositol 1,4,5-triphosphate (IP_3) in neutrophils

2×10^6 neutrophils suspended in 0.2 ml of cell culture medium (GIT™; Nihon Seiyaku, Tokyo, Japan) were placed in conical plastic tubes. After pretreatment with various concentrations of *S*-carboxymethylcysteine for 1 h, the cells were exposed to 10^{-6} M of FMLP for 15 s. We preliminary determined that IP_3 production in neutrophils reached a maximum after 15 s of cell exposure to FMLP. Incubation was terminated with an equal volume of ice-cold 15% (v/v) trichloroacetic acid. After the centrifugation at $2000 \times g$ for 15 min at 4 °C, the supernatants were extracted three times with 10 volumes of water-saturated diethyl ether. The aqueous phase was transferred to a 1.5-ml Eppendorf centrifuge tube and neutralized to pH 7.5 with $NaHCO_3$. The amount of IP_3 was measured using a competitive radiobinding assay system (Amersham Scientific Products, McGraw Park, IL).

2.5. Measurement of 1,2-diacylglycerol in neutrophils

2×10^6 neutrophils suspended in 0.2 ml of GIT™ were placed in conical plastic tubes. After pretreatment with various concentrations of *S*-carboxymethylcysteine for 1 h, the cells were exposed to 10^{-6} M of FMLP for 5 min. The reactions were quenched with 3 ml of ice-cold chloroform/methanol (1:2). Sufficient 1.0 M NaCl was added to bring the aqueous volume to 0.8 ml. The monophase was

mixed, and 1.0 ml of chloroform and 1.0 ml of 1.0 M NaCl were added to break up the phases. Following centrifugation at $5000 \times g$ for 2 min, the lower phase was transferred to the polypropylene tube and evaporated to dryness under nitrogen. The amount of 1,2-diacylglycerol was measured using a radioenzymatic assay system (Amersham).

2.6. Measurement of cellular cyclic AMP levels

2×10^6 neutrophils suspended in 0.2 ml of GIT™ were placed in conical plastic tubes. After pretreatment with various concentrations of *S*-carboxymethylcysteine for 1 h, the cells were centrifuged and resuspended in HBSS containing 0.2% bovine serum albumin. Cells were then incubated with 10^{-6} M of FMLP for 5 min. The reaction was terminated by boiling the samples for 2 min. The cell debris was removed by centrifugation, and the supernatant containing total cyclic AMP was dried under vacuum. The extracts were redissolved in 0.05 M acetate buffer containing 0.02% bovine serum albumin (pH 5.8). Total cellular cyclic AMP was determined by enzyme immunoassay using a kit (Amersham).

2.7. Measurement of phosphatidyl inositol-specific phospholipase C activity in vitro

The activity of phosphatidyl inositol-specific phospholipase C was determined as the solubilizing activity for membrane-bound acetylcholinesterase (Taguchi and Ikezawa, 1987). The reaction mixture, which contained 10 mM Tris–HCl buffer, pH 7.4, 144 mM NaCl, 0.05% BSA, 0.1 unit/ml of phosphatidyl inositol-specific phospholipase C (Sigma), and 6.25 mg/ml of membrane-bound acetylcholinesterase (Sigma), was incubated for 10 min at 30 °C with various concentrations of *S*-carboxymethylcysteine. An equal volume of 200 mM sodium acetate solution (pH 4.5) was then added, and the mixture was placed on ice for 15 min. After centrifugation, 0.05 ml of supernatant was transferred to a new conical tube, and 1.45 ml of ATI/DTNB solution (1 mM acetylthiocholine iodide with 0.2 mM 5,5′-dithio-bis 2-Nitrobenzoic acid) in 100 mM sodium phosphate containing 0.1% Triton X-100 (pH 7.4, Sigma), was then added. After a 10-min incubation at 30 °C, the reaction was terminated by 0.1 ml of 0.001% phenylmethylsulfonyl fluoride diluted in isopropanol. The reaction was followed spectrophotometrically at 412 nm. Results are presented as percentages of the positive control values.

2.8. Statistical analysis

Results are presented as means ± S.E.M. Data were analyzed by a standard one-way analysis of variance in combination with Duncan's multiple comparison test. A level of $P < 0.05$ was accepted as statistically significant.

3. Results

3.1. Inhibition of neutrophil chemotaxis by S-carboxymethylcysteine

We first examined the viability of neutrophils after incubation with *S*-carboxymethylcysteine to evaluate whether *S*-carboxymethylcysteine causes direct cell toxicity. The cell viability always exceeded 98%, as determined by trypan blue exclusion, which did not differ from before incubation with *S*-carboxymethylcysteine.

We then evaluated the effects of *S*-carboxymethylcysteine on neutrophil chemotactic activity toward FMLP. A total of 657 ± 78 neutrophils were observed in 10 high-performance fields (hpf) by a chemotactic chamber toward 10^{-6} M FMLP (Fig. 2). Migrated neutrophils were significantly decreased to 299 ± 13 and 144 ± 10 in 10 hpf after the incubation with 10 and 100 μg/ml of *S*-carbox-

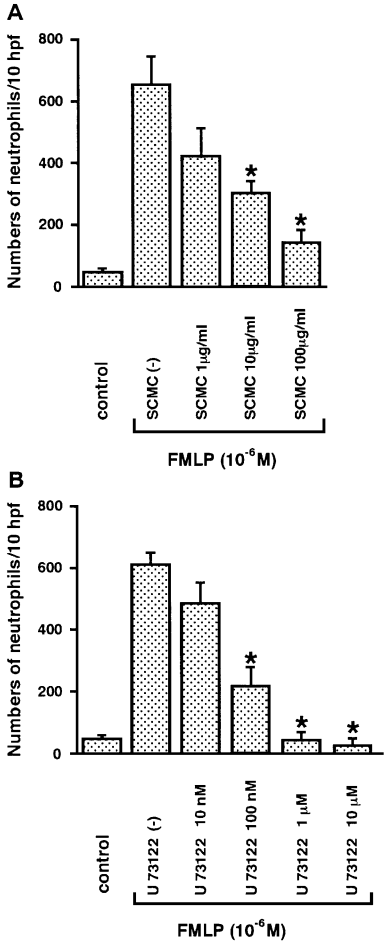

Fig. 2. The effects of (A) *S*-carboxymethylcysteine (SCMC) and (B) U73122 on neutrophil chemotactic activity toward 10^{-6} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). Results are presented as the number of migrated neutrophils in 10 high-performance fields and represent the mean ± S.E.M. of eight donors. The control represents the values obtained from neutrophils unstimulated with FMLP. * Significantly different than values from the neutrophils untreated with *S*-carboxymethylcysteine (SCMC (−)) ($P<0.05$).

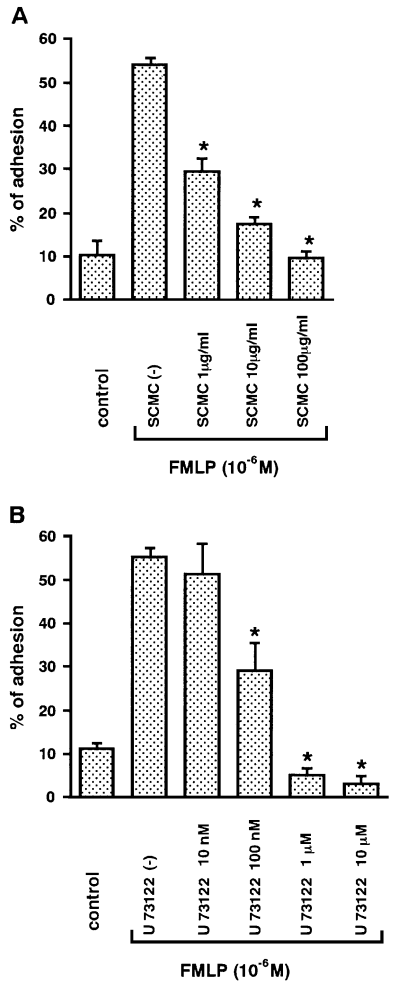

Fig. 3. The effects of (A) *S*-carboxymethylcysteine (SCMC) and (B) U73122 on neutrophil adhesion to pulmonary vascular endothelium. Neutrophils were stimulated with 10^{-6} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), and pulmonary vascular endothelium was pretreated with 15 U/ml of interleukin-1β and 25 ng/ml of tumor necrosis factor-α for 3 h. Results are presented as a percentage of adhered neutrophils and represent the mean ± S.E.M. of eight donors. The control represents the values obtained from neutrophils unstimulated with FMLP. * Significantly different than values from the neutrophils untreated with *S*-carboxymethylcysteine (SCMC (−)) ($P<0.05$).

ymethylcysteine, respectively (Fig. 2A). The number of migrated neutrophils was also decreased dose-dependently after the incubation with U73122, an inhibitor of phosphatidyl inositol-specific phospholipase C (Fig. 2B). Pretreatment of neutrophils with BAPTA-AM did not alter the number of migrated neutrophils from the values of untreated controls (data not shown).

3.2. Inhibition of neutrophil adhesion to endothelial cells by *S*-carboxymethylcysteine

After the stimulation of neutrophils with 10^{-6} M FMLP, the neutrophil adhesion rate increased significantly in controls (10.2 ± 3.4% to 54.0 ± 2.0%) (Fig. 3). Preincubation with more than 1 μg/ml of *S*-carboxymethylcysteine significantly inhibited neutrophil adhesion to pulmonary vascular endothelial cells, with the neutrophils showing adhesion rates of 29.6 ± 2.9%, 17.6 ± 1.3%, and 9.8 ± 1.4% at *S*-carboxymethylcysteine concentrations of 1, 10, and 100 μg/ml, respectively (Fig. 3A). Neutrophil adhesion to pulmonary vascular endothelial cells was also inhibited dose dependently after the incubation with U73122 (Fig. 3B). Pretreatment of neutrophils with BAPTA-AM did not alter the neutrophil adhesion rate from the values of untreated controls (data not shown).

3.3. Effects of *S*-carboxymethylcysteine on IP_3 production in neutrophils

After the stimulation of neutrophils with 10^{-6} M FMLP, IP_3 production was significantly increased from a value of 0.17 ± 0.08 to 1.40 ± 0.31 pmol/10^6 cells (Fig. 4). Preincubation with more than 10 μg/ml of *S*-carboxymethylcysteine significantly decreased IP_3 production in FMLP-stimulated neutrophils to 0.50 ± 0.10 pmol/10^6 cells (10 μg/ml of *S*-carboxymethylcysteine), and 0.33 ± 0.08 pmol/10^6 cells (100 μg/ml of *S*-carboxymethylcysteine), respectively (Fig. 4).

3.4. Effects of *S*-carboxymethylcysteine on 1,2-diacylglycerol production in neutrophils

After the stimulation of neutrophils with 10^{-6} M FMLP, 1,2-diacylglycerol production was significantly increased from a value of 13.3 ± 3.0 to 61.7 ± 17.3 pmol/10^6 cells (Fig. 5). Preincubation with 100 μg/ml of *S*-carboxymethyl-

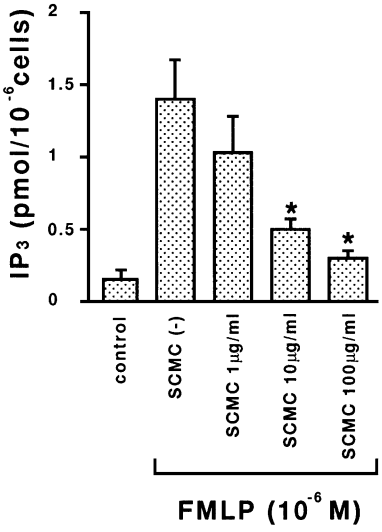

Fig. 4. The effects of *S*-carboxymethylcysteine (SCMC) on the production of inositol 1,4,5-triphosphate (IP_3) in neutrophils stimulated with 10^{-6} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). Results represent the mean ± S.E.M. of eight donors. The control represents the values obtained from neutrophils unstimulated with FMLP. * Significantly different than values from the neutrophils untreated with *S*-carboxymethylcysteine (SCMC (−)) ($P<0.05$).

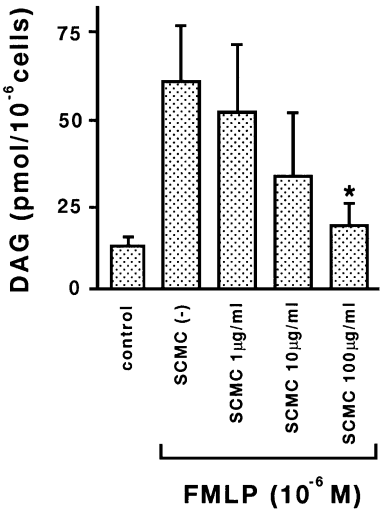

Fig. 5. The effects of *S*-carboxymethylcysteine (SCMC) on the production of 1,2-diacylglycerol (DAG) in neutrophils stimulated with 10^{-6} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). Results represent the mean ± S.E.M. of eight donors. The control represents the values obtained from neutrophils unstimulated with FMLP. * Significantly different than values from the neutrophils untreated with *S*-carboxymethylcysteine (SCMC (−)) ($P<0.05$).

cysteine significantly decreased 1,2-diacylglycerol production in FMLP-stimulated neutrophils to 19.3 ± 7.3 pmpl/10^6 cells (Fig. 5).

3.5. Effects of S-carboxymethylcysteine on cellular cyclic AMP levels

Cellular cyclic AMP levels were significantly elevated in neutrophils after the stimulation with 10^{-6} M FMLP compared with unstimulated controls (Fig. 6). No concentration of *S*-carboxymethylcysteine significantly altered cellular cyclic AMP levels in FMLP-stimulated neutrophils (Fig. 6).

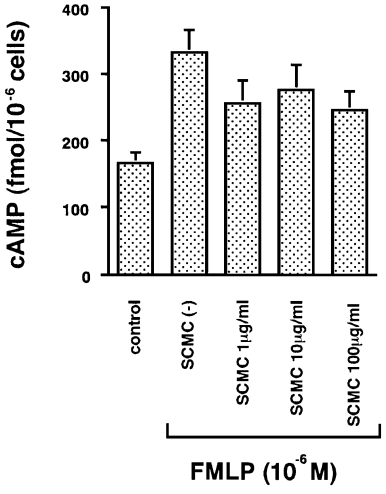

Fig. 6. The effects of *S*-carboxymethylcysteine (SCMC) on cellular cAMP levels in neutrophils stimulated with 10^{-6} M *N*-formyl-methionyl-leucyl-phenylalanine (FMLP). Results represent the mean ± S.E.M. of eight donors. The control represents the values obtained from neutrophils unstimulated with FMLP.

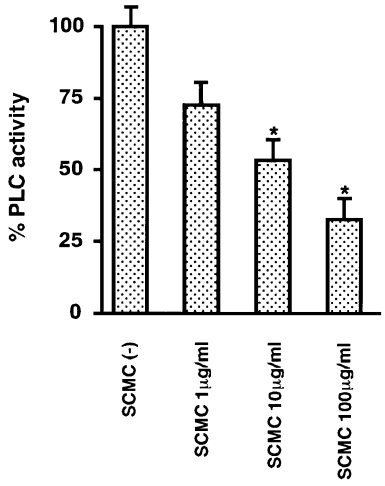

Fig. 7. The effects of *S*-carboxymethylcysteine (SCMC) on phosphatidyl inositol-specific phospholipase C (PI-PLC) activity in vitro. Results are presented as the percentage of phosphatidyl inositol-specific phospholipase C activity without *S*-carboxymethylcysteine (SCMC (−)), and represent the mean ± S.E.M. of four different experiments. * Significantly different than values from the activity untreated with *S*-carboxymethylcysteine (SCMC (−)) ($P<0.05$).

3.6. Effects of S-carboxymethylcysteine on phosphatidyl inositol-specific phospholipase C activity in a cell-free system

To confirm whether *S*-carboxymethylcysteine directly affects phosphatidyl inositol-specific phospholipase C, the effects of *S*-carboxymethylcysteine on phosphatidyl inositol-specific phospholipase C enzymatic activity in vitro were investigated. *S*-carboxymethylcysteine caused a concentration-dependent inhibition of phosphatidyl inositol-specific phospholipase C activity (Fig. 7), with 28.1 ± 7.0%, 47.0 ± 7.3%, and 66.4 ± 7.2% inhibition being observed with 1, 10, and 100 μg/ml of *S*-carboxymethylcysteine, respectively (Fig. 7).

4. Discussion

In the present study, incubation of *S*-carboxymethylcysteine with neutrophils was found to cause a concentration-dependent inhibition of both neutrophil chemotactic activity against FMLP and FMLP-mediated neutrophil adhesion to pulmonary vascular endothelial cells. *S*-carboxymethylcysteine belongs to a family of thiol-containing mucolytics. *N*-acetylcysteine, the same type of mucolytic, has also shown an inhibition of neutrophil activation including superoxide anion generation (Villagrasa et al., 1997). The inhibitory effects of *N*-acetylcysteine on neutrophil activation are considered to be mediated by its antioxidant effect. *N*-acetylcysteine has been shown to

interact directly with oxidants, and it may also exert its antioxidant effects indirectly by facilitating GSH biosynthesis (Cotgreave, 1997). In contrast, the antioxidant effects of *S*-carboxymethylcysteine have not been demonstrated until now because, unlike *N*-acetylcysteine, its sulphydryl is not free.

FMLP-induced signal transduction mechanisms have previously been investigated. A common feature of FMLP receptor-mediated neutrophil activation consists of a G protein-dependent activation of phosphatidyl inositol-specific phospholipase C that catalyzes the hydrolysis of phosphatidylinositol bisphosphate (PIP_2) to produce IP_3 and 1,2-diacylglycerol (Di Virgilio et al., 1985; Verghese et al., 1986; Dillon et al., 1987). IP_3 mediates the release of calcium from an intracellular pool, and 1,2-diacylglycerol activates protein kinase C, thus activating several functions of neutrophils (Berridge, 1984, 1986; Burnham et al., 1989; O'Flaherty et al., 1989). The present study demonstrates that preincubation of neutrophils with *S*-carboxymethylcysteine inhibits both IP_3 and 1,2-diacylglycerol production after FMLP stimulation, suggesting that *S*-carboxymethylcysteine attenuates neutrophil activation at least in part through an inhibition of phosphatidyl inositol-specific phospholipase C activity. Our finding that *S*-carboxymethylcysteine inhibits phosphatidyl inositol-specific phospholipase C activity in a cell-free system also supports this explanation. Moreover, the findings that FMLP-mediated neutrophil activation is similarly inhibited by U73122, an inhibitor of phosphatidyl inositol-specific phospholipase C, demonstrates that the inhibition of phosphatidyl inositol-specific phospholipase C activity results in the inhibition of biological responses of neutrophils.

In the present study, both IP_3 production after FMLP stimulation and phosphatidyl inositol-specific phospholipase C activity in a cell-free system were inhibited by more than 10 μg/ml of *S*-carboxymethylcysteine. However, FMLP-mediated neutrophil adhesion to pulmonary vascular endothelial cells was inhibited by lower concentrations of *S*-carboxymethylcysteine (1 μg/ml) than those necessary to inhibit phosphatidyl inositol-specific phospholipase C activity. The additional mechanisms of *S*-carboxymethylcysteine in the inhibition of neutrophil adhesion remain to be elucidated. It has been reported that the CD11/CD18 family of leukocyte adhesion glycoproteins play a critical role in stimulated neutrophil adherence to endothelium (Albelda et al., 1994; Tonnesen et al., 1989; Zimmerman and McIntyre, 1988). In the CD11/CD18 family, lymphocyte function-associated antigen-1 (CD11a/CD18) is important as a cell-surface ligand for intercellular adhesion molecule-1, an adhesion molecule induced on the endothelial surface in response to the activation with interleukin-1β, and tumor necrosis factor-α (Tonnesen, 1989; Norris, 1990). Mac-1 (CD11b/CD18) has also been shown to be induced significantly after stimulation with chemotactic factors, including FMLP, and thus may be primarily involved in neutrophil adhesion to endothelium (Anderson et al., 1986; Zimmerman and McIntyre, 1988). We are investigating the effects of *S*-carboxymethylcysteine on the expressions of lymphocyte function-associated antigen-1 and Mac-1 on neutrophils because our preliminary study demonstrated that *S*-carboxymethylcysteine partially reduces neutrophil expression of CD11a and CD11b after stimulation with FMLP (data not shown).

S-carboxymethylcysteine inhibits neutrophil chemotaxis in vitro not only after stimulation with FMLP, but also after stimulation with leukotriene B_4 and zymosan-activated serum (Hirata and Ohashi, 1995). Like FMLP, the actions of leukotriene B_4 and complement C5a, a major chemotactic component of zymosan-activated serum, are mediated by their specific cell-surface receptors. The activation of neutrophils by both leukotriene B_4 and C5a is also regulated by receptor-coupled G protein-dependent activation of phosphatidyl inositol-specific phospholipase C as well as its induction of IP_3 formation (Mong et al., 1986; Gerard and Gerard, 1994; Yokomizo et al., 1997). Therefore, the finding that *S*-carboxymethylcysteine inhibits phosphatidyl inositol-specific phospholipase C activity does not contradict current hypotheses regarding the inhibitory mechanisms of *S*-carboxymethylcysteine in relation to leukotriene B_4 and zymosan-activated serum-mediated neutrophil activation.

Enhanced cAMP levels were observed in neutrophils stimulated with FMLP in the present study. Transient increases in intracellular cAMP levels have been previously observed in neutrophils after stimulation with chemotactic factors (Smolen et al., 1980; Simchowicz et al., 1980; Verghese et al., 1985). Unlike the hormonal agonists causing G protein-mediated activation of adenylate cyclase, chemoattractants, including FMLP, increase cAMP levels by mobilizing intracellular calcium and subsequent activation of adenylate cyclase (Verghese et al., 1985). In the present study, *S*-carboxymethylcysteine did not affect cAMP levels in stimulated neutrophils, suggesting that *S*-carboxymethylcysteine is not involved in the activation of adenylate cyclase, an enzyme that is not coupled directly to chemoattractant receptors in human neutrophils (Verghese et al., 1985).

It is generally thought that the rise of intracellular Ca^{2+} levels regulates several cell responses. However, it is not known whether the rise of phospholipase C-mediated intracellular Ca^{2+} is necessary for the migration and adhesion of neutrophils. In the present study, pretreatment with BAPTA-AM, an intracellular Ca^{2+}-chelator, did not affect neutrophil migration and adhesion after stimulation with FMLP. Similarly, Kuijpers et al. (1992) have demonstrated that neither neutrophil adherence to nor migration across confluent monolayers of endothelial cells were inhibited by using sufficient amount of BAPTA-AM to inhibit a rapid rise of intracellular Ca^{2+}. Perez et al. (1989) also noted that chelation of intracellular Ca^{2+} did not affect the ability of neutrophils to respond chemotactically.

Serum concentrations of *S*-carboxymethylcysteine have been tested in healthy adults following oral administration of 1.5 g of *S*-carboxymethylcysteine (De Schutter et al.,

1988), with a maximal concentration of 13.9 μg/ml at 2 h post-dosing and a mean concentration of 0.87 μg/ml persisting at 10 h after oral administration. Together with our present results, these findings suggest that the concentrations of *S*-carboxymethylcysteine obtained by routine clinical use (500 mg p.o., three times a day) may not be sufficient to inhibit neutrophil activation. Although some additional issues may arise when the administered dose of *S*-carboxymethylcysteine increases to produce concentrations that can inhibit neutrophil activation, the properties of *S*-carboxymethylcysteine observed in the present study can generally be expected when it is used as a regulatory agent of neutrophilic inflammation in clinical applications.

References

Albelda, S.M., Smith, C.W., Ward, P.A., 1994. Adhesion molecules and inflammatory injury. FASEB J. 8, 504–512.

Amatrude, T.T., Graonic, S.D., Holmes, R., Perez, H.D., 1995. Signal transduction by the formyl peptide receptor. J. Biol. Chem. 270, 28010–28013.

Anderson, D.C., Miller, L.J., Schmalstieg, F.C., Rothlein, R., Springer, T.A., 1986. Contributions of the Mac-1 glycoprotein family to adherence-dependent granulocyte functions: structure–function assessments employing subunit-specific monoclonal antibodies. J. Immunol. 137, 15–27.

Berridge, M.J., 1984. Inositol triphosphate and diacyl glycerol as second messengers. Biochem. J. 220, 345–360.

Berridge, M.J., 1986. Intracellular signalling through inositol triphosphate and diacyl glycerol. Biol. Chem. Hoppe-Seyler 367, 447–456.

Burnham, D.N., Tyagi, S.R., Uhlinger, D.J., Lambeth, J.D., 1989. Diacyl glycerol generation and phosphoinositide turnover in human neutrophils: effects of particulate versus soluble stimuli. Arch. Biochem. Biophys. 15, 345–353.

Cotgreave, I.A., 1997. *N*-acetylcysteine: pharmacological considerations and experimental and clinical applications. Adv. Pharmacol. 38, 205–227.

Dallegri, F., Ottonello, L., 1997. Tissue injury in neutrophilic inflammation. Inflamm. Res. 46, 382–391.

De Schutter, J.A., Van Der Weken, G., Van Den Bossche, W., De Moerloose, P., 1988. Determination of *S*-carboxymethylcysteine in serum by reversed-phase ion-pair liquid chromatography with column switching following pre-column derivatization with *o*-phthalaldehyde. J. Chromatogr. 428, 301–310.

Dillon, S.B., Murray, J.J., Verghese, M.W., Snyderman, R., 1987. Regulation of inositol phosphate metabolism in chemoattractant-stimulated human polymorphonuclear leukocytes. J. Biol. Chem. 262, 11546–11552.

Di Virgilio, F., Vicentini, L.M., Treves, S., Riz, G., Pozzan, T., 1985. Inositol phosphate formation in fMet-Leu-Phe-stimulated human neutrophils does not require an increase in the cytosolic free Ca^{2+} concentration. Biochem. J. 229, 361–367.

Gallin, J.I., 1988. Phagocytic cells: disorders of function. In: Gallin, J.I., Goldstein, I.M., Snyderman, R. (Eds.), Inflammation, Basic Principles and Clinical Correlates. Raven Press, New York, pp. 493–511.

Gerard, C., Gerard, N.P., 1994. C5a anaphylatoxin and its seven transmembrane-segment receptor. Annu. Rev. Immunol. 12, 775–808.

Hirata, Y., Ohashi, M., 1995. Effect of *S*-carboxymethylcysteine (S-CMC) on bleomycin-induced lung injury in mice. Kiso to Rinsho 29, 3661–3666.

Kuijpers, T.W., Hoogerwerf, M., Roos, D., 1992. Neutrophil migration across monolayers of resting or cytokine-activated endothelial cells. J. Immunol. 148, 72–77.

Lew, P.D., 1990. Receptors and intracellular signaling in human neutrophils. Am. Rev. Respir. Dis. 141, S127–S131.

Mong, S., Chi-Rosso, G., Miller, J., Hoffman, K., Razgaitis, K.A., Bender, P., Crooke, S.T., 1986. Leukotriene B4 induces formation of inositol phosphates in rat peritoneal polymorphonuclear leukocytes. Mol. Pharmacol. 30, 235–242.

Norris, D.A., 1990. Cytokine modulation of adhesion molecules in the regulation of immunologic cytotoxicity of epidermal targets. J. Invest. Dermatol. 95, 111S–120S.

O'Flaherty, J.T., Jacobson, P.D., Redman, J.F., Rossi, A.G., 1989. Translocation of protein kinase C in human polymorphonuclear neutrophils. J. Biol. Chem. 265, 9146–9152.

Palmblad, J., 1984. The role of granulocytes in inflammation. Scand. J. Rheumatol. 13, 163–172.

Perez, H.D., Elfman, F., Marder, S., Lobo, E., Ives, H.E., 1989. Formyl peptide induced chemotaxis of human polymorphonuclear leukocytes does not require either marked changes in cytosolic calcium or specific granule discharge. J. Clin. Invest. 83, 1963–1970.

Simchowicz, L., Fischbein, L.C., Spilberg, I., Atkinson, J.P., 1980. Induction of a transient elevation in intracellular levels of adenosine-3′,5′-cyclic monophosphate by chemotactic factors: an early event in human neutrophil activation. J. Immunol. 124, 1482–1491.

Smith, J.A., 1994. Neutrophils, host defense, and inflammation: a double-edged sword. J. Leukoc. Biol. 56, 672–686.

Smolen, J.E., Korchak, H.M., Weissmann, G., 1980. Increased levels of cyclic adenosine-3′,5′-monophosphate in human polymorphonuclear leukocytes after surface stimulation. J. Clin. Invest. 65, 1077–1085.

Taguchi, R., Ikezawa, H., 1987. Properties of bovine erythrocyte acetylcholinesterase solubilized by phosphatidylinositol-specific phospholipase C1. J. Biochem. 102, 803–811.

Tonnesen, M.G., 1989. Neutrophil–endothelial cell interactions: mechanisms of neutrophil adherence to vascular endothelium. J. Invest. Dermatol. 93, 53S–58S.

Tonnesen, M.G., Anderson, D.C., Springer, T.A., Knedler, A., Avdi, N., Henson, P.M., 1989. Adherence of neutrophils to cultured human microvascular endothelial cells: stimulation by chemotactic peptides and lipid mediators and dependence upon the Mac-1, LFA-1, p150, 95 glycoprotein family. J. Clin. Invest. 83, 637–646.

Vartio, T., Seppa, H., Vaheri, A., 1981. Susceptibility of soluble and matrix fibronectin to degradation by tissue proteinases, mast cell chymase, and cathepsin G. J. Biol. Chem. 256, 471–477.

Verghese, M.W., Fox, K., McPhan, L.C., Snyderman, R., 1985. Chemoattractant-elicited alteration of cAMP levels in human polymorphonuclear leukocytes requires a Ca^{++}-dependent mechanism which is independent of transmembrane activation of adenylate cyclase. J. Biol. Chem. 260, 6769–6775.

Verghese, M.W., Smith, C.D., Snyderman, R., 1986. Role of guanine nucleotide regulatory protein in polyphosphoinositide degradation and activation of phagocytic leukocytes by chemoattractants. J. Cell. Biochem. 32, 59–69.

Villagrasa, V., Cortijo, J., Marti-Cabrera, M., Ortiz, J.L., Berto, L., Esteras, A., Bruseghini, L., Morcillo, E.J., 1997. Inhibitory effects of *N*-acetylcysteine on superoxide anion generation in human polymorphonuclear leukocytes. J. Pharm. Pharmacol. 49, 525–529.

Weiss, S.J., 1989. Tissue destruction by neutrophils. N. Engl. J. Med. 320, 365–376.

Yokomizo, T., Izumi, T., Chang, K., Takuwa, Y., Shimizu, T., 1997. A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis. Nature 387, 620–624.

Zimmerman, G.A., McIntyre, T.M., 1988. Neutrophil adherence to human endothelium in vitro occurs by CDw18 (Mol, Mac-1/LFA-1/GP150, 95) glycoprotein-dependent and -independent mechanisms. J. Clin. Invest. 81, 531–537.