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The inhibitory effect of ambroxol on hypochlorous acid-induced tissue damage and respiratory burst of phagocytic cells

Yong Cho, Yoon Young Jang, Eun Sook Han, Chung Soo Lee *

Department of Pharmacology, College of Medicine, Chung-Ang University, Seoul 156-756, South Korea

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Abstract

Ambroxol (100 μM and 1 mM) and the thiols (all 1 mM), glutathione, tiopronin and cysteine, significantly attenuated the myeloperoxidase, H_2O_2 and Cl^- system-caused destruction of α_1-antiproteinase and the HOCl-induced destruction of collagen, whereas they did not affect the elastase-induced destruction of collagen. Glutathione, tiopronin and cysteine almost completely decomposed both HOCl and H_2O_2, while ambroxol up to 1 mM did not show a scavenging action on H_2O_2. Ambroxol (1 to 100 μM) and 1 mM thiol compounds markedly inhibited the HOCl-induced alteration of elastase activity. Thiol compounds significantly attenuated the HOCl production caused by degraded immunoglobulin G-activated neutrophils. Ambroxol depressed superoxide and H_2O_2 production induced by degraded immunoglobulin G-activated neutrophils and by lipopolysaccharide-activated alveolar macrophages in a dose-dependent manner. The results show that ambroxol may interfere with oxidative tissue damage and decrease proteolytic tissue destruction by attenuation of oxidative stress-induced inactivation of α_1-antiproteinase through both decomposition of HOCl and inhibition of the respiratory burst in phagocytic cells.

Keywords: Ambroxol; Hypochlorous acid; α_1-Antiproteinase destruction; Respiratory burst

1. Introduction

α_1-Antiproteinase is inactivated by various oxidizing substances and reactive oxygen species discharged by activated phagocytic cells (Weiss, 1989). Hypochlorous acid (HOCl) produced by the myeloperoxidase–H_2O_2–Cl^- system inactivates plasma α_1-antiproteinase and can activate neutrophil metalloproteinase, such as collagenase. Damaged α_1-antiproteinase and free neutrophil elastase are detected in bronchial exudates in adult respiratory distress syndrome. Inactivation of α_1-antiproteinase is suggested to promote elastase-induced tissue injury. Elastase is thought to be involved in the tissue injury seen in respiratory distress syndrome (Cochrane et al., 1983), cystic fibrosis (Suter et al., 1986), emphysema (Knight et al., 1997) and arthritis (Virca et al., 1984).

Endogenous and exogenous antioxidants are expected to protect tissue components against oxidants. Ambroxol, trans-4 [(2-amino-3,5-dibromobenzyl) amino] cyclohexanol HCl, is known to promote bronchial secretion and is used as an expectorant (Disse, 1987). Ambroxol has been shown to improve the clinical course of respiratory distress syndrome, including bronchopulmonary dysplasia (Wauer et al., 1992), and to reduce postoperative pulmonary complications (Fegiz, 1991). It has been reported that ambroxol has an antioxidant action (Nowak et al., 1994a) and some anti-inflammatory effect (Bianchi et al., 1990; Park et al., 1999). It inhibits lipid peroxidation of lung tissue induced by heat and H_2O_2 (Nowak et al., 1994b) and protects hyaluronic acid and collagen against oxidative attack (Koh et al., 1998). However, it does not prevent lung damage caused by paraquat and hyperoxia (Nemery et al., 1992) and does not inhibit the peroxidation of liver lipids. Ambroxol is known to scavenge hydroxyl radicals, while its ability to remove superoxide anion and H_2O_2 is uncertain (Nowak et al., 1994a; Gillissen et al., 1997).

Cellular sulfhydryl groups play a major role in the maintenance of membrane integrity and cell functions. Sulfhydryl groups can provide protection against oxidants, including HOCl. N-Acetylcysteine inhibits oxidative degradation of deoxyribose (Vanderbist et al., 1996). Glutathione (GSH) significantly decreases the cytotoxicity of tumor necrosis factor-α on insulinoma cells (Cavallo et al.,

* Corresponding author. Tel.: +82-2-820-5659; fax: +82-2-815-3856; e-mail: leecs@cau.ac.kr

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1997) and completely inhibits lactoferrin-B-induced apoptosis in human monocytic leukemic cells (Yoo et al., 1997). However, GSH also has a pro-oxidant action and could evoke cellular damage by the formation of thiyl radicals (Halliwell and Gutteridge, 1989). Exogenous GSH enhances myoglobin toxicity in mouse renal proximal tubular (HK-2) cells (Zager and Burkhart, 1998). Cysteine promotes H_2O_2-induced proximal tubular cell injury but decreases myoglobin toxicity in the same cells.

In the present study, in order to explore the protective role of ambroxol in HOCl-mediated tissue injury, the effect of ambroxol on inactivation or destruction of α_1-antiproteinase induced by the myeloperoxidase–H_2O_2–Cl^- system and on collagen degradation caused by elastase or HOCl were investigated, and the action was compared with that of the thiol compounds, GSH, tiopronin and cysteine. An experiment was performed in order to investigate by which mechanism they exert protective effects. Their effects on oxidant production induced by degraded immunoglobulin G-activated neutrophils and by lipopolysaccharide-activated alveolar macrophages were examined.

The present data indicate that ambroxol may interfere with oxidative tissue damage and decrease proteolytic tissue destruction by attenuation of oxidative stress-induced inactivation of α_1-antiproteinase through both scavenging of HOCl and inhibition of the respiratory burst in phagocytic cells.

2. Materials and methods

2.1. Drugs

α_1-Antiproteinase (α_1-antitrypsin from human plasma), human leukocyte elastase, myeloperoxidase (from human leukocyte), glucose oxidase (from *Aspergillus niger*), cartilage collagen (from bovine trachea), ambroxol (AMB), glutathione (reduced form, GSH), *N*-(2–mercaptopropionyl)-glycine (tiopronin), cysteine, dimethyl sulfoxide (DMSO), human immunoglobulin G, lipopolysaccharide (from *Escherichia coli*), taurine, *N*-methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitroanilide, polyoxyethylene 23 lauryl ether (Brij–35), cytochalasin B, ferricytochrome *c*, scopoletin, horseradish peroxidase and Ficoll–Hypaque solution were purchased from Sigma-Aldrich (St. Louis, MO, USA). NaOCl and H_2O_2 were obtained from Junsei Chemical. Other chemicals were of analytical grade.

2.2. Electrophoretic analyses of α_1-antiproteinase and collagen

α_1-Antiproteinase (25 μg/50 μl) was treated with 1 U/50 μl myeloperoxidase, 300 μM H_2O_2 and 150 mM NaCl for 2 h in the presence or absence of ambroxol (or thiol compounds). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970) on 10% acrylamide slab gels. SDS-PAGE was carried out at 40 mA for 60 min.

Cartilage collagen (100 μg/50 μl) was treated either with 5 μM HOCl for 1 h or with 25 mU/50 μl elastase in the presence or absence of the stated compounds for 72 h. SDS-PAGE was performed on 5% acrylamide slab gels and was carried out at 40 mA for 75 min. The gels shown in Fig. 2 were analyzed by using Image gauge 3.11 program in FUSIFILM BAS-2500 system, Japan.

2.3. Preparation and assay of hypochlorous acid

HOCl was prepared immediately before use by adjusting NaOCl to pH 6.2 with diluted H_2SO_4 (Green et al., 1985). The concentration of HOCl was determined using a molar extinction coefficient of 142 at 291 nm.

2.4. Measurement of hypochlorous acid production

HOCl production in reaction mixtures (0.5 ml) containing 100 mU/ml myeloperoxidase, 200 μM H_2O_2, 150 mM NaCl and 15 mM taurine was assayed by measuring the formation of taurine chloramine (Shacter et al., 1991). The reaction was terminated by addition of 5 μl of 1 mg/ml catalase and 10 μl of 1 M KI. The absorbance was read at 350 nm, and the amount of HOCl produced was estimated using an extinction coefficient of $2.29 \times 10^4 M^{-1} cm^{-1}$ for OCl^-.

HOCl generation by activated neutrophils was measured in a reaction mixture consisting of 2×10^6 cells/ml, 0.5 mg/ml degraded immunoglobulin G, 15 mM taurine and Hanks' balanced salt solution (HBSS), pH 7.4. The reaction was terminated by addition of catalase and KI, sequentially, and the absorbance was measured.

2.5. Scan of absorbance spectrum at various wavelengths

Ambroxol (100 μM) was treated with 2.5 μM HOCl, and then the absorbance change was recorded at wavelengths ranging between 230 and 400 nm.

2.6. Measurement of hydrogen peroxide production

H_2O_2 production was carried out in a reaction mixture containing 20 mM glucose, 25 mU/ml glucose oxidase, 150 mM KCl and 50 mM NaH_2PO_4 buffer, pH 7.4. The reaction medium was mixed with stopping solution (25 mg/ml of potassium biphthalate, 2.5 mg/ml NaOH, 82.5 mg/ml potassium iodide and 0.25 mg/ml ammonium molybdate), and the absorbance was measured at 350 nm (Koh et al., 1998).

2.7. Measurement of elastase activity

Elastase activity was assayed using the specific substrate, *N*-methoxysuccinyl–Ala–Ala–Pro–Val–*p*-nitro-

anilide, at 37°C (Wasil et al., 1987; Janusz and Hare, 1994). The assay medium (0.5 ml) consisted of 0.2 mM substrate, 0.1% Brij-35, 20 μg α_1-antiproteinase, HOCl, 0.5 M NaCl and 50 mM NaH_2PO_4, pH 7.4. Elastase activity was measured as an increase in absorbance at 410 nm.

2.8. Preparation of human neutrophils

Neutrophils were prepared from fresh whole human blood, anticoagulated with 10% acid-citrate-dextrose, by dextran sedimentation, hypotonic lysis of erythrocytes and Ficoll–Hypaque density centrifugation (Markert et al., 1984). The neutrophils were suspended in Dulbecco's phosphate-buffered saline (PBS), pH 7.4 at a concentration of 1×10^7/ml. Final suspensions of neutrophils consisted of about 97% neutrophils as judged by Wright–Giemsa staining, and viability was more than 98% as judged by trypan blue dye exclusion.

Human immunoglobulin G was heated to degradation at 63°C for 30 min. After neutrophils were pretreated with cytochalasin B (5 μg/ml for 10^7 cells), the assay for respiratory burst was performed.

2.9. Isolation of rat alveolar macrophages

Sprague–Dawley rats weighing about 250 g were anesthetized by intraperitoneal injection of 40 mg/kg pentobarbital sodium. A tracheal cannula was inserted through an incision in the neck, and 6 ml of cooled Ca^{2+}, Mg^{2+}-free PBS, pH 7.4, was instilled in the lung via a syringe attached to the cannula. Cell suspensions were treated with hypotonic solution to lyse red blood cell. The cell pellets were resuspended in Ca^{2+}, Mg^{2+}-free HBSS, placed on a Ficoll–Hypaque gradient and centrifuged at 400 g for 45 min at 4°C. Macrophages were collected from the interphase of the gradient. The cells were washed with HBSS and were suspended in the same solution (Witz et al., 1987).

2.10. Measurement of superoxide production by phagocytic cells

The superoxide-dependent reduction of ferricytochrome *c* was measured by the method of Markert et al. (1984). The reaction mixtures in plastic microfuge tubes contained 2×10^6 neutrophils, 75 μM ferricytochrome *c*, degraded immunoglobulin G and HBSS, pH 7.4, in a total volume of 1.0 ml, and the reactions were performed at 37°C for 15 min. The reaction was stopped by placing the tubes in melting ice, and the cells were rapidly pelleted by centrifugation at $800 \times g$ for 5 min at 4°C. The supernatants were taken, and the amount of reduced cytochrome *c* was calculated using an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} at 550 nm (Cohen and Chovaniec, 1978).

Alveolar macrophages (3×10^5 cells/well) were treated with 1 μg/ml lipopolysaccharide in 100 μl of a reaction mixture containing ferricytochrome *c* and HBSS, pH 7.4, in 96-well microplates at 37°C for 4 h. The absorbance was measured at 550 nm in a microplate reader.

2.11. Measurement of hydrogen peroxide production by phagocytic cells

H_2O_2 produced by activated neutrophils was assayed by measuring the change in scopoletin fluorescence (Root et al., 1975). The reaction mixture contained 2×10^6 neutrophils, 2.5 μM scopoletin, 5 μg/ml horseradish peroxidase and HBSS buffer in a total volume of 1.0 ml. After preincubation for 5 min at 37°C with compounds, the reaction was initiated by addition of immunoglobulin G and lasted for 15 min. The decrease in scopoletin fluorescence by H_2O_2 produced was measured at the wavelengths of excitation, 343 nm, and emission, 460 nm. The concentration of H_2O_2 was determined using H_2O_2 as standard.

In order to eliminate the influence of a long incubation on scopoletin fluorescence, macrophage-induced H_2O_2 production was measured using phenol red (Pick and Mizell, 1981). Alveolar macrophages (3×10^5 cells/well) were treated with 1 μg/ml lipopolysaccharide in reaction mixture (100 μl) consisting of 0.1 mg/ml phenol red, 0.1 mg/ml horseradish peroxidase and HBSS, pH 7.4, in 96-well microplates at 37°C for 4 h. The reaction was stopped by addition of 10 μl of 1 N NaOH, and the absorbance was measured at 610 nm.

2.12. Data analysis

The results obtained in various experiments were analyzed for level of significance using the Student's *t*-test for paired data. The *p* value indicates that data were significantly different from data obtained without addition of ambroxol or thiol compound. Data represent means ± S.D.

3. Results

3.1. Protective effects of ambroxol and thiol compounds on HOCl-induced damage of α_1-antiproteinase

HOCl appears to be implicated in tissue damage at sites of inflammation. Damaged α_1-antiproteinase is detected in fluids recovered from inflamed sites. Intact α_1-antiproteinase showed one major band (Mr = 65,000) and minor bands. Leukocyte myeloperoxidase exhibited two bands of approximate Mr = 71,000 and Mr = 59,000. Cleavage of

α_1-antiproteinase was induced by the myeloperoxidase system (myeloperoxidase, H_2O_2 and Cl^-). Destruction of the antiproteinase was enhanced with increasing incubation times, and the mass size of the protein bands decreased. The damaging effect of the myeloperoxidase system on α_1-antiproteinase under our reaction conditions was weaker than the oxidizing action of 1 μM HOCl (data not shown). Fig. 1 shows the protective effects of ambroxol and the thiols, GSH and cysteine, against the destruction of α_1-antiproteinase caused by the myeloperoxidase system. Ambroxol (100 μM) partially protected α_1-antiproteinase against the damaging action of myeloperoxidase system. Ambroxol and thiol compounds at 1 mM concentration almost completely inhibited the myeloperoxidase system-induced destruction of α_1-antiproteinase.

3.2. Effects of ambroxol, GSH, tiopronin and cysteine on destruction of collagen by HOCl or elastase

The destruction of cartilage collagen was produced by addition of HOCl or neutrophil elastase. Cartilage collagen was resistant to HOCl, and treatment of collagen with 5 μM HOCl resulted in partial destruction compared to that seen with the antiproteinase. Destruction of cartilage collagen was chiefly detected in two protein bands, approximate Mr = 127,000 and Mr = 108,000. These bands had a relative density of 2,593,000 psl and 980,000 psl, respectively, in the Image gauge 3.11 program. In the present study, HOCl caused a 33% decrease in the relative density of the major band and a 35% decrease in the minor band. HOCl-induced collagen destruction was attenuated by 100 μM ambroxol and 1 mM of GSH, tiopronin and cysteine (Fig. 2). Under these experimental conditions, at the concentrations used, ambroxol exhibited little protective effect (18% and 30% at major and minor bands), while thiol compounds had an apparent protective action (GSH: 88% and 73% at major and minor bands, tiopronin: 73% and 46% and cysteine: 86% and 36%) against the oxidative action of HOCl. Neutrophil elastase caused proteolytic cleavage of cartilage collagen. As shown in Fig. 3, in contrast to the oxidant, the elastase-induced collagen de-

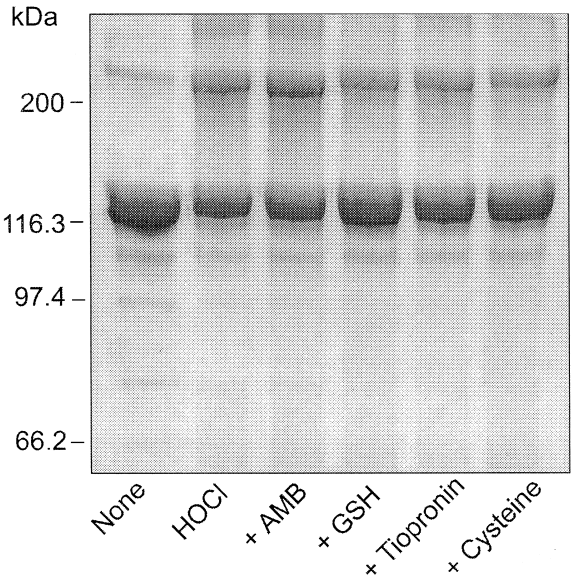

Fig. 2. Attenuation of HOCl-induced destruction of collagen by ambroxol and thiol compounds. Collagen (100 μg/50 μl) was treated with 5 μM HOCl in the presence of 100 μM ambroxol and 1 mM thiols.

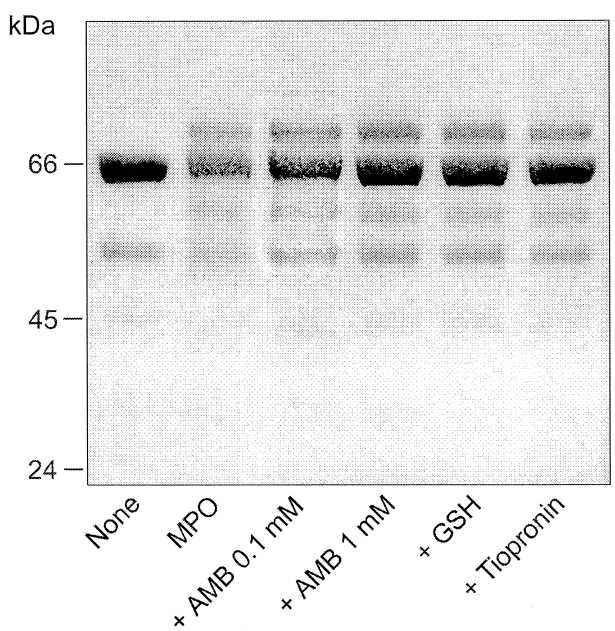

Fig. 1. Protection of myeloperoxidase system-induced destruction of α_1-antiproteinase by ambroxol and thiol compounds. Antiproteinase (25 μg/50 μl) was treated with myeloperoxidase system in the presence of ambroxol (100 μM and 1 mM) and 1 mM of thiol compounds for 2 h.

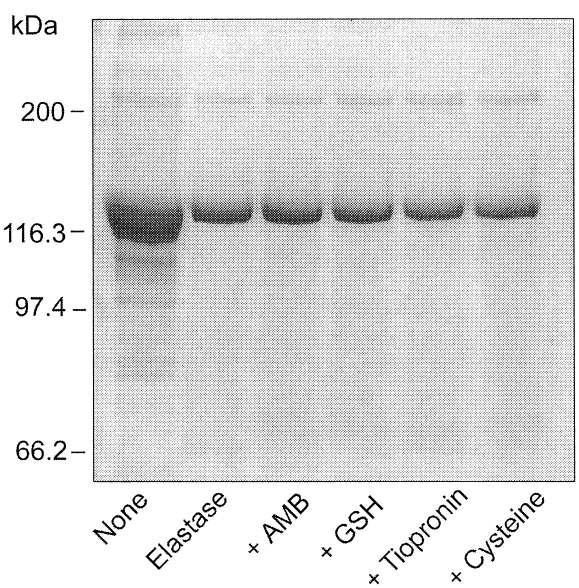

Fig. 3. Effects of ambroxol and thiol compounds on elastase-caused destruction of collagen. Elastase (25 mU/50 μl) was added to a reaction mixture containing collagen and stated compounds, and the reaction lasted for 72 h.

struction did not respond to equal concentrations of ambroxol and thiol compounds.

3.3. Decomposing actions of ambroxol and thiol compounds on myeloperoxidase-produced HOCl

GSH is one of the preferred biological substrates of myeloperoxidase-derived HOCl, and becomes oxidized by it (Winterbourn and Brennan, 1997). HOCl production induced by 100 mU/ml myeloperoxidase, 200 μM H_2O_2 and 150 mM NaCl exhibited a peak value (27.16 ± 1.23 nmol, $n = 3$) at 5 min after addition of myeloperoxidase, and the amount produced gradually declined with increasing incubation times. Fig. 4 shows the decomposing action of thiol compounds on HOCl produced by the myeloperoxidase system. HOCl production was decreased by addition of GSH in a dose-dependent manner. Preincubation of GSH, tiopronin and cysteine (1 mM) completely depressed myeloperoxidase-evoked HOCl production. The direct scavenging action of thiol compounds on prepared HOCl was measured at 291 nm (Fig. 5) and was confirmed by the addition of DMSO, a scavenger of HOCl. The stated amounts of GSH, tiopronin and cysteine removed HOCl, with a resultant decrease in absorbance. The scavenging effect of ambroxol on HOCl was difficult to assay because the interaction of ambroxol with HOCl resulted in an absorbance increase in the detection wavelength range of HOCl. However, the elevated absorbance gradually decreased with increasing reaction times.

The decomposing action of ambroxol on HOCl was investigated as the change in absorbance spectrum of ambroxol due to possible complex formation. Ambroxol had two absorbance peaks at 240 nm and 310 nm, and HOCl had a peak at 291 nm. Fig. 6 shows that ambroxol (100 μM) treated with 2.5 μM HOCl had an altered absorbance spectrum, and the ambroxol–HOCl complex showed a broad increase in absorbance in the wavelength range of 230–320 nm.

Hydrogen peroxide is considered as a precursor of more reactive oxygen species and forms metal ion–H_2O_2 complexes that cause oxidative damage. The scavenging actions of the compounds used on glucose/glucose oxidase-produced H_2O_2 or diluted H_2O_2 were examined. In order to eliminate the effect of ambroxol on glucose oxidase activity, its direct decomposing action on H_2O_2 was investigated. As shown in Fig. 7, in both reaction mixtures, GSH, tiopronin and cysteine (1 mM) completely decomposed H_2O_2 which was scavenged by catalase. In contrast, ambroxol up to 1 mM did not exhibit a scavenging action on H_2O_2 during a 30 min incubation.

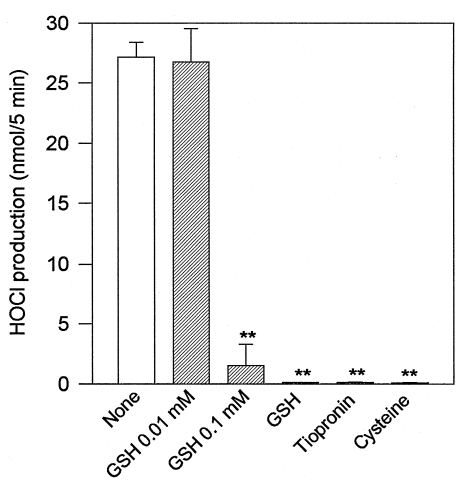

Fig. 4. Effects of thiol compounds on myeloperoxidase-induced HOCl production. HOCl produced in a reaction mixture containing 100 mU/ml myeloperoxidase, 200 μM H_2O_2, 150 mM NaCl, 15 mM taurine and 1 mM thiols was measured. Data represent means ± S.D., $n = 3$. $^{**}P < 0.01$ significantly different from no addition of thiol compounds.

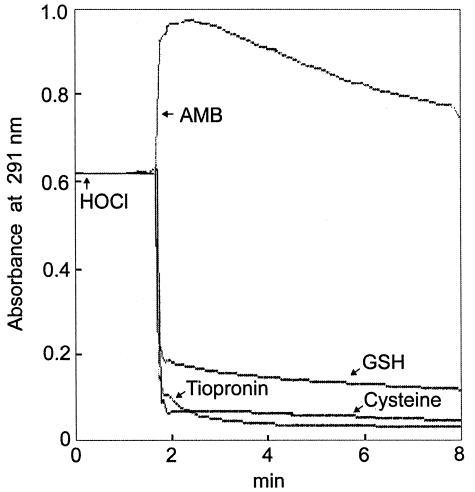

Fig. 5. Decomposing actions of thiol compounds on HOCl. Ambroxol (100 μM) or 1 mM thiol compounds reacted with 5 μM HOCl, and absorbance change was read at 291 nm.

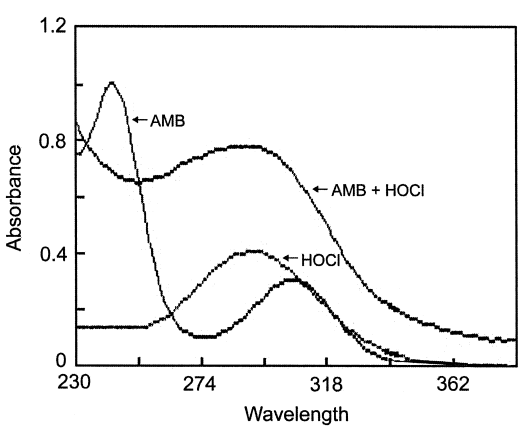

Fig. 6. Change of absorbance spectrum of ambroxol at variable wavelengths. Ambroxol (100 μM) were treated with 2.5 μM HOCl, and absorbance change was recorded at wavelengths between 230–400 nm.

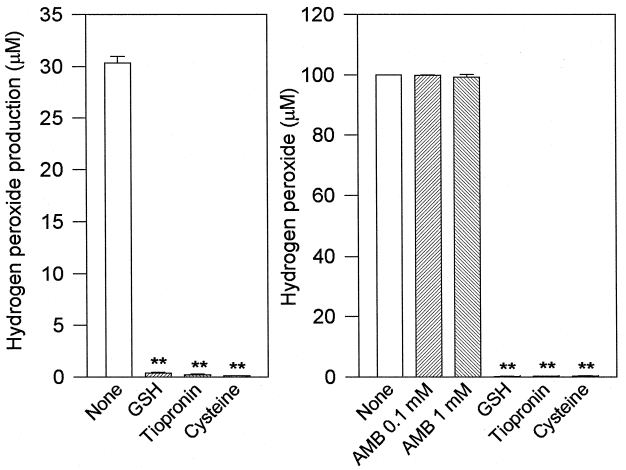

Fig. 7. Decomposing effects of ambroxol and thiol compounds on hydrogen peroxide. Ambroxol (100 μM) was mixed with 100 μM H_2O_2 and 1 mM thiol compounds reacted either with H_2O_2 produced by 20 mM glucose and 25 mU/ml glucose oxidase or with 100 μM H_2O_2. The remaining H_2O_2 was measured as described in Materials and methods. Data represent means ± S.D., $n = 3$. $^{**}P < 0.01$ significantly different from no addition of ambroxol and thiol compounds.

3.4. Inhibition of HOCl-induced alteration of elastase activity by ambroxol and thiol compounds

Neutrophil elastase is known to be involved in the tissue destruction associated with a number of inflammatory diseases. Neutrophil elastase exposed to 1 μM HOCl showed a 40.2% reduction in activity. In the reaction mixture without the antiproteinase, ambroxol affected the elastase activity attenuated by HOCl. Fig. 8 shows that ambroxol decreased the HOCl-induced inactivation of elastase in a dose-dependent manner, and at 100 μM, complete inhibition was seen. Thiol compounds also protected α_1-antiproteinase against oxidative attack by HOCl. The effects of thiol compounds on elastase activity were examined in a reaction mixture containing HOCl, antiproteinase and elastase. GSH, tiopronin and cysteine (1 mM) significantly inhibited the activation of elastase due to the HOCl-induced inactivation of α_1-antiproteinase.

3.5. Depressant effect of ambroxol on oxidant production by phagocytic cells

Phagocytosis of immunoglobulin G by neutrophils leads to stimulation of the respiratory burst (Ahn et al., 1996). Heat-aggregated immunoglobulin G (0.5 mg/ml) stimulated neutrophils and produced 2.15 ± 0.06 nmol/2×10^6 cells/15 min of HOCl ($n = 4$). The decomposing action of thiol compounds on HOCl produced by activated neutrophils was studied. As can be seen in Fig. 9, thiol compounds (1 mM) significantly decreased the amount of HOCl produced by 20.5% to 29.8%. In contrast to the cell-free system, complete or pronounced decomposing effects of thiol compounds were not seen.

Ambroxol caused color development in the HOCl-measuring reaction mixture. Thus, instead of HOCl, the effect of ambroxol on superoxide anion and H_2O_2 production by activated neutrophils was examined. Ambroxol depressed the stimulated respiratory burst in degraded immunoglobulin G-activated neutrophils in a dose-dependent manner (Table 1). At 100 μM, superoxide production was inhibited by 43.3%,and H_2O_2 production was depressed by 64.3%. The inhibitory action of ambroxol on the respiratory burst was also seen in lipopolysaccharide-activated rat alveolar macrophages. Lipopolysaccharide-stimulated

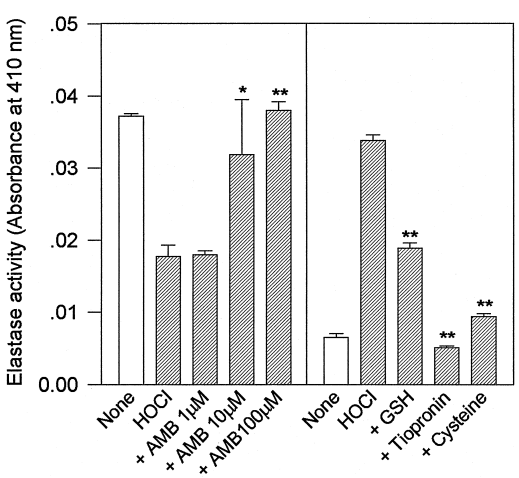

Fig. 8. Effects of ambroxol and thiol compounds on elastase activity. The effects of the compounds on elastase activity were assayed in a reaction mixture containing 1 μM HOCl, 40 μg/ml α_1-antiproteinase, 40 mU/ml elastase and 0.2 mM substrate. Antiproteinase was omitted from the ambroxol assay mixture. Data represent means ± S.D., $n = 3$. $^{*}P < 0.05$, $^{**}P < 0.01$ significantly different from no addition of ambroxol or thiol compounds.

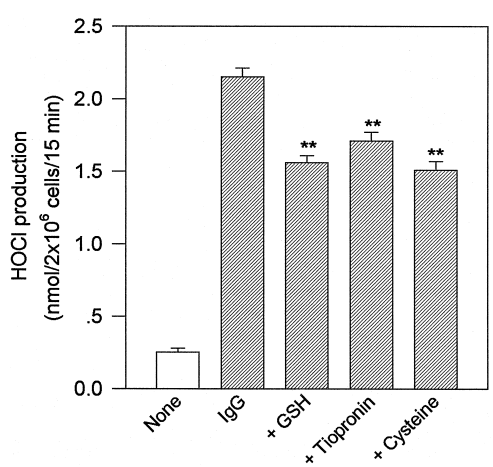

Fig. 9. Effects of thiol compounds on HOCl produced by activated neutrophils. Human neutrophils (2×10^6 cells/ml) were stimulated with 0.5 mg/ml degraded immunoglobulin G in the presence of 1 mM thiols. Data represent means ± S.D., $n = 4$. $^{**}P < 0.01$ significantly different from no addition of thiol compounds.

Table 1
Inhibitory effect of ambroxol on stimulated superoxide and hydrogen peroxide production in activated phagocytic cells
Neutrophils (2×10^6 cells) were stimulated with 0.5 mg/ml degraded immunoglobulin G, and alveolar macrophages (3×10^5 cells) were activated by addition of 1 μg/ml lipopolysaccharide in the presence of ambroxol. Data represent means ± S.D., $n = 4–5$.

	Neutrophils		Macrophages	
	Superoxide anion (nmol)	H_2O_2 (nmol)	Superoxide anion (nmol)	H_2O_2 (nmol)
No addition	17.46 ± 1.32	8.00 ± 0.31	3.46 ± 0.39	1.67 ± 0.18
Ambroxol 1 μM	17.52 ± 1.12	7.68 ± 0.73	2.69 ± 0.43	1.23 ± 0.11[a]
10 μM	15.48 ± 1.20	6.04 ± 0.23[b]	1.89 ± 0.41[b]	0.61 ± 0.16[b]
100 μM	9.82 ± 1.28[b]	2.86 ± 0.13[b]	1.14 ± 0.33[b]	0.52 ± 0.11[b]

[a] $P < 0.05$
[b] $P < 0.01$ Significantly different from no addition of ambroxol by Student's *t*-test.

superoxide and H_2O_2 production was inhibited by addition of ambroxol in a dose-dependent manner.

4. Discussion

Reactive oxygen species appear to be implicated in tissue damage in various pathologic conditions. The destruction of bronchial tissue and joint components associated with inflammation is ascribed in part to the role of free radicals. In inflammatory situations, reactive oxidants are released from phagocytic cells infiltrating sites of inflammation. Activated neutrophils form the powerful oxidant, HOCl, by the catalyzing action of myeloperoxidase. The highly reactive HOCl reacts with most biological molecules, degrading structural proteins, inactivating the major plasma protease inhibitor, α_1-antiproteinase, and activating neutrophil collagenase (Weiss, 1989). Thus, HOCl may promote tissue damage by facilitating elastase- and collagenase-induced proteolysis at inflammatory sites. In inflammatory conditions, including pulmonary emphysema and rheumatoid arthritis, many neutrophils accumulate in body fluids and release oxidants (Halliwell et al., 1988). However, human extracellular fluids, such as plasma and synovial fluid, contain low amounts of the antioxidant defense enzymes, glutathione peroxidase, superoxide dismutase and catalase. Thus, oxidants are not detoxified and react with tissue components to cause damage.

α_1-Antiproteinase is capable of passing through vascular membranes into tissues in inflammatory situations (Travis and Salvesen, 1983). It is considered to play an important role in controlling tissue damage caused by the massive quantities of proteinases released from phagocytic cells. α_1-Antiproteinase accounts for about 90% of the elastase-inhibitory capacity of human serum, and its inactivation may greatly potentiate tissue damage. When antiproteinase was exposed to prepared HOCl and the myeloperoxidase–H_2O_2–Cl^- system, it was destroyed and lost its inhibitory effect on neutrophil elastase activity. HOCl cleaved electrophoretic bands of α_1-antiproteinase, and the mass size of the major and minor protein bands was decreased. Concentrations of HOCl above 60 μM are reported to inhibit antiproteinase activity by 95–100% in reaction mixtures containing pancreatic elastase, while under 10 μM it has little inhibitory effect (Wasil et al., 1987). In the present study, when the antiproteinase was mixed with 1 μM HOCl for 10 min, its inhibitory effect on neutrophil elastase was decreased by 34.8%. The response of neutrophil elastase to the inhibitory action of α_1-antiproteinase may be different from that of pancreatic elastase.

Ambroxol has been shown to inhibit H_2O_2-induced lipid peroxidation in lung tissues, while it does not protect lung tissues against paraquat and peroxidation of liver lipids. Thus, the biological effect of ambroxol on tissue components under oxidative stress is variable. GSH is known to have both antioxidant ability and pro-oxidant action. Myeloperoxidase-derived HOCl is able to oxidize many biological molecules, especially thiol groups. N-Acetylcysteine is suggested as an effective thiol in the maintenance of the protease/antiprotease balance (Vanderbist et al., 1996). This thiol has also been demonstrated to inhibit the production of HOCl and hydroxyl radicals by stimulated neutrophils (Gressier et al., 1994). Ambroxol and the thiol compounds, GSH, tiopronin and cysteine, significantly attenuated the destruction or inactivation of α_1-antiproteinase either by prepared HOCl or by the myeloperoxidase–H_2O_2–Cl^- system. They protected antiproteinase and elastase against the reactive oxidant HOCl. The result indicates that these compounds protect α_1-antiproteinase against the oxidative attack of HOCl.

The damaging action of HOCl on tissue components was examined in cartilage collagen. Cartilage collagen was more resistant to HOCl than α_1-antiproteinase and was partially destroyed by treatment with 5 μM HOCl. The protective effects of ambroxol and the thiol compounds on HOCl-caused collagen degradation suggest that they could protect antiproteinases, including α_1-antiproteinase, against oxidative attack and then interfere with the proteolytic tissue damage caused by elastase in inflammatory diseases. Proteolytic damage of articular cartilage is an important feature of inflammatory joint diseases (Virca et al., 1984).

Elastase has proteolytic activity on connective tissue macromolecules (Janusz and Doherty, 1991). Neutrophil elastase destroyed cartilage collagen, and the damage increased with increasing incubation times. However, in contrast to oxidative damage, ambroxol, GSH, tiopronin and cysteine did not protect against elastase-caused collagen degradation. Thus, compounds protect antiproteinases through their oxidant scavenging actions and in this way have a protective action against tissue injury associated with inflammation.

Stimulated phagocytic cells discharge reactive oxidants, such as superoxide anion and H_2O_2, and lysosomal enzymes. The secretory products play an important role in host defense mechanisms and inflammatory responses and are involved in tissue destruction. Neutrophils use approximately 80% of H_2O_2 generated to produce HOCl by means of the myeloperoxidase system (Ottonello et al., 1994). Thiol compounds (1 mM) had a strong decomposing action on H_2O_2, and the action was finished within 5 min. They also had inhibitory effects on the generation of H_2O_2 by the glucose and glucose oxidase system. Meanwhile, ambroxol up to 1 mM did not show a decomposing action on H_2O_2, in agreement with previous report (Gillissen et al., 1997). GSH, tiopronin and cysteine had a scavenging action on prepared HOCl and on H_2O_2 generated by the myeloperoxidase system. However, under the present experimental conditions, it is difficult to measure a direct scavenging action of ambroxol on HOCl because the interaction of ambroxol with HOCl produced an absorbance increase in the detection wavelength range of HOCl. However, the decomposing action of ambroxol on HOCl may be postulated on the basis of the absorbance decrease after the initial elevation, which increased with increasing reaction times, and on the basis of the change in absorbance spectrum of ambroxol due to a complex formation. The decomposing action of ambroxol on HOCl could also be inferred from its protective effect on α_1-antiproteinase against oxidative attack by HOCl. In contrast to ambroxol, the thiol compounds caused HOCl to decompose markedly, and interaction did not cause an absorbance increase. Thiol compounds could protect antiproteinases against oxidative attack of HOCl by causing H_2O_2 decomposition as well as by direct scavenging of HOCl.

The uptake of immunoglobulin G by phagocytic cells plays a critical role in the host response to infection and immune complex-mediated diseases, such as systemic lupus erythematosus (Raghavan and Bjorkman, 1996). At sites of inflammation, phagocytosis of degraded immunoglobulin G by neutrophils leads to stimulation of the respiratory burst and to the release of lysosomal enzymes. Neutrophils exposed to degraded immunoglobulin G discharge superoxide anion, H_2O_2 and HOCl. The depressant action of ambroxol on HOCl production induced by immunoglobulin G-stimulated neutrophils could be inferred from its significant inhibitory effect on the respiratory burst. The reported scavenging effect of ambroxol on superoxide anion is weak and is statistically insignificant (Gillissen et al., 1997). Ambroxol has been demonstrated to show a decomposing action on H_2O_2 (Nowak et al., 1994a), to attenuate H_2O_2 production in the rat liver (Piotrowski et al., 1996) and to inhibit lipid peroxidation in lung homogenates induced by H_2O_2 (Nowak et al., 1994b). However, a decomposing effect of ambroxol on H_2O_2 has not been investigated (Gillissen et al., 1997). In the present study, ambroxol up to 1 mM did not show a decomposing action on H_2O_2. Our previous report demonstrates an inhibitory action of ambroxol on *N*-formyl–methionyl–leucyl–phenylalanine-induced myeloperoxidase release in human neutrophils (Park et al., 1999). These findings indicate that ambroxol has a depressant action on the respiratory burst in neutrophils. It is postulated that ambroxol inhibits HOCl production through its depressant action on superoxide and H_2O_2 production by degraded immunoglobulin G-activated neutrophils. The inhibitory action of ambroxol on the respiratory burst was also seen in lipopolysaccharide-activated alveolar macrophages. GSH is necessary for the expression of neutrophil responses and is regenerated during the activation of neutrophils (Ogino et al., 1997). Thus, it is reasonable that thiol compounds do not show an inhibitory action on neutrophil responses, such as respiratory burst and degranulation. The attenuation of HOCl production in immunoglobulin G-activated neutrophils by the thiol compounds seen in this study appears to be due to their scavenging actions on H_2O_2 and HOCl.

In conclusion, ambroxol appears to protect tissues against oxidative damage by a mechanism of action different from that of thiol compounds. Ambroxol may interfere with oxidative tissue damage and decrease proteolytic tissue destruction by attenuating the oxidative stress-induced inactivation of α_1-antiproteinase as a result of its scavenging action on HOCl and inhibition of the respiratory burst in phagocytic cells.

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