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Protective Action of Ambroxol on the Oxidative Damages of Lipid, Hyaluronic Acid and Collagen

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Abstract – Ambroxol is thought to have antioxidant ability and some antiinflammatory effect. Effect of ambroxol on the oxidative damages of lipid, collagen and hyaluronic acid was examined. Fe^{2+} (10 μM) and 100 μM ascorbate-induced lipid peroxidation of liver microsomes was inhibited by 10 and 100 μM ambroxol, 30 μg/ml catalase and 10 mM DABCO but was not affected by 30 μg/ml SOD and 10 mM DMSO. A 10 and 100 μM ambroxol and 10 mM DABCO inhibited the peroxidative action of 10 μM Fe^{3+}, 160 μM ADP and 100 μM NADPH on microsomal lipids, whereas inhibitory effects of 30 μg/ml SOD, 30 μg/ml catalase and 10 mM DMSO were not detected. The degradation of hyaluronic acid caused by 10 μM Fe^{2+}, 500 μM H_2O_2 and 100 μM ascorbate was inhibited by 10 and 100 μM ambroxol, 30 μg/ml catalase, 10 mM DMSO and 10 mM DABCO, while 30 μg/ml SOD did not show any effect. The cartilage collagen degradation caused by 30 μM Fe^{2+}, 500 μM H_2O_2 and 200 μM ascorbate was prevented by 100 μM ambroxol. H_2O_2 and OH · were scavenged by ambroxol, whereas O_2^- was not removed by it. Ambroxol (100 μM) and 1 mM cysteine reduced DPPH to 1,1-diphenyl-2-picrylhydrazine. In conclusion, ambroxol may inhibit the oxidative damages of lipid, hyaluronic acid and collagen by its scavenging action on oxidants, such as OH · and probably iron-oxygen complexes and exert antioxidant ability.

Keywords□ ambroxol, antioxidant, oxidative damages of lipid, hyaluronic acid, collagen

Reactive oxygen species and oxidant-derived species including lipid peroxides have been shown to be implicated in the tissue damage that occurs in certain diseases, such as rheumatoid arthritis (Merry *et al.*, 1989), postischemic tissue damage (Downey, 1990), atherosclerosis (Witztum and Steinberg, 1991) and diabetes (Wolff *et al.*, 1991) and in drug toxicities (Trush *et al.*, 1982). An increased production of reactive oxygen species in pathologic and drug toxicity states contributes to the damage of tissue components. Oxidoreduction of metal ions, particularly iron, is considered to play a central role in the tissue destruction, and iron catalyzes effectively the tissue damage by oxidants (Halliwell and Gutteridge, 1989b).

The oxidants react with joint components to cause a destruction of articular cartilage, bone erosion and impairment of joint function (Greenward and Moy, 1980; Merry *et al.*, 1989). In addition, iron is deposited within ferritin and hemosiderin in the synovial membranes (Blake *et al.*, 1981; Biemond *et al.*, 1986). Exposure of iron protein to the oxidants releases iron to synovial fluid and into synovial lining cells. Iron effectively catalyzes formation of highly reactive oxygen species, such as OH · and singlet oxygen($^1O_2$) (Halliwell, 1978; Halliwell and Gutteridge, 1989a). Iron-oxygen complexes have also been proposed as a causative agent for the oxidative tissue damages.

Ambroxol, 4-[[(2-amino-3,5-dibromophenyl)methyl]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), reduce postoperative pulmonary complications (Fegiz, 1991) and reduce concentration of uric acid in plasma (Oosterhuis *et al.*, 1993). Ambroxol is thought to have antioxidant ability (Nowak *et al.*, 1994a) and some antiinflammatory effect (Stockley *et al.*, 1988; Bianchi *et al.*, 1990). It has been reported that ambroxol inhibits lipid peroxidation of lung tissue by heat and H_2O_2 (Nowak *et al.*, 1994b) and protects linoleic acid from OH · -induced peroxidative damage (Nowak *et al.*, 1994a). However, ambroxol does not prevent lung damage by paraquat and hyperoxia (Nemery *et al.*, 1992).

To determine the antioxidant mechanism of ambroxol,

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its effect on the oxidative damages of lipid, collagen and hyaluronic acid caused by reactive oxygen species and metal ion, iron was studied. The scavenging action of ambroxol on reactive oxygen metabolites was also examined.

MATERIALS AND METHODS

Chemicals

Ambroxol, superoxide dismutase (from bovine blood, SOD), catalase (from bovine liver), dimethyl sulfoxide (DMSO), 1,4-diazabicyclo (2,2,2) octane (DABCO), hyaluronic acid (Grade III from human umbilical cord), tracheal cartilage collagen, xanthine, xanthine oxidase, ascorbic acid, NADPH, 2-thiobarbituric acid (TBA), ferricytochrome c, 2-α deoxyribose and 1,1-diphenyl-2-picryl-hydrazil (DPPH) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals were of analytical grade.

Preparation of Rat Liver Microsomes

The microsomal fraction was prepared from the rat liver according to Appel *et al.* (1981). Male Sprague-Dawley rats weighing about 150 g were used. Livers were placed in ice cold buffer I (0.25 M sucrose, 0.02 M Tris-HCl, 0.5 mM EDTA, pH 7.4) and homogenized in 4 vol. of buffer I using polytron homogenizer (Brinkman, Model PT-20). After removal of cell debris, nuclei and mitochondria by centrifugation for 10 min at 500, 1,000 and 10,000 g, the microsomal fraction was pelleted by centrifugation at 100,000 g for 60 min. The resultant pellets were suspended in homogenizing buffer II (0.12 M KCl, 0.05 M Tris-HCl, pH 7.4) and centrifuged at 20,000 g for 20 min. The supernatants were centrifuged at 100,000 g for 60 min, and the pellets were resuspended in buffer II. Protein concentration was determined by the method of Lowry *et al.* (1951) and stored at -70 ℃.

Measurement of Lipid Peroxidation

Lipid peroxidation of microsomes was estimated from measuring malondialdehyde concentration by thiobarbituric acid method (Gutteridge, 1981; Gutteridge *et al.*, 1982). Liver microsomes (0.2 mg protein/ml) were contained in the reaction mixture (1.0 ml) consisting of 150 mM KCl, 10 μM FeSO4 plus 100 μM ascorbate (or 10 μM $FeCl_3$, 160 μM ADP and 100 μM NADPH) and 50 mM sodium phosphate buffer, pH 7.4. Reaction was conducted at 37 ℃ for 30 min. The absorbance was measured at 532 nm. The concentration of malondialdehyde was expressed as n mol/mg protein using the molar extinction coefficient of 1.52×10^5/M/cm (Placer *et al.*, 1966).

Assay of Xanthine Oxidase Activity

A 10 μl of one tenth diluted xanthine oxidase was placed in a cuvette which contains 2 ml of mixture containing 0.2 mM xanthine and 50 mM sodium phosphate buffer, pH 7.4. The absorbance of urate produced was read at 290 nm. By use of the molar extinction coefficient of urate (1.24×10^4/M/cm), the amount of urate was calculated. One unit of xanthine oxidase activity was defined as 1 μM urate produced per minute (Greenward and Moy, 1979).

Viscometry

Viscosity of hyaluronic acid was measured using a modified Cannon capillary viscometer, which is regulated by vacuum pressure. The reaction mixtures contained 1 mg/ml hyaluronic acid, 150 mM KCl and 50 mM potassium phosphate buffer, pH 7.5. The viscosity change was measured at 25 ℃ and expressed as a flow time (sec).

Electrophoretic Analysis

Cartilage collagen (200 μg/100 μl) was incubated with 30 μM $FeSO_4$, 500 μM H_2O_2 and 200 μM ascorbic acid in the presence of ambroxol for 6 h at 37 ℃. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Lammeli (1970) on 5% acrylamide slab gels. SDS-PAGE was carried out at 40 mA for 75 min.

Assay of Superoxide Anion

Superoxide anion produced was measured by reduction of ferricytochrome c. Measurement of superoxide anion production was done in 2 ml of reaction mixtures contained 75 μM ferricytochrome c, 0.2 mM xanthine, 10.5 mU/ml xanthine oxidase, ambroxol, 150 mM KCl and 50 mM sodium phosphate buffer, pH 7.4. The reduction of ferricytochrome c by xanthine and xanthine oxidase was read at 550 nm, and the amount of reduced cytochrome c was determined using an extinction coefficient of $2.1\times10^4M^{-1}\ cm^{-1}$ (Cohen and Chovaniec, 1978).

Assay of H_2O_2 Decomposition

The concentration of H_2O_2 was measured by the method of Allen *et al.* (1952). The reaction mixtures contained, in a final volume of 1.0 ml, 120 mM KCl, 0.1 mM H_2O_2, ambroxol, 10 μM sodium azide and 50 mM Tris-HCl, pH 7.4. After reaction, 2.5 ml of stopping solution (25 mg/ml of potassium biphthalate, 2.5 mg/ml NaOH, 82.5 mg/ml potassium iodide and 0.25 mg/ml am-

monium molybdate) was added to the above mixture, and absorbance change was read at 350 nm.

Assay of the Thiobarbituric Acid Reactivity of 2-α Deoxyribose

Amount of hydroxyl radical generated was estimated from the thiobarbituric acid (TBA) reactivity of 2-α deoxyribose (Gutteridge, 1981; Halliwell and Gutteridge, 1981). The reaction mixtures contained 2 mM 2-α deoxyribose, 50 μM $FeCl_3$, 50 μM EDTA, 500 μM H_2O_2, and 100 μM ascorbate (or 0.2 mM xanthine and 42.1 mU/ml xanthine oxidase), 150 mM KCl and 50 mM sodium phosphate buffer, pH 7.4 in a final volume of 1.0 ml. Reaction was conducted at 37 ℃ for 30 min. The fluorescence was read at the wavelengths of excitation, 532 nm and emission, 553 nm.

Assay of Reduction of Free Radical DPPH

The change in absorbance produced by interaction of ambroxol with DPPH was measured by the method of previously reported paper (Constantin *et al.*, 1990). The reaction mixtures contained 60 μM DPPH, ambroxol and 50 mM Tris-HCl, pH 7.4. The absorbance was read at 517 nm.

Data Analysis

The results obtained in various experiments were analysed for level of significance using the Student's *t*-test.

RESULTS

Effect of Ambroxol on Lipid Peroxidation by Iron and Reducing Agents

The antioxidant ability of ambroxol on nonenzymatic lipid peroxidation of microsomes was investigated. Iron catalyzes production of reactive oxygen intermediates and stimulates the oxidative tissue damages. Fig. 1 shows that ambroxol significantly inhibited lipid peroxidation of liver microsomes by 10 μM Fe^{2+} and 100 μM ascorbate in a dose dependent fashion, and at a concentration of 100 μM, a 74.9% of inhibition was detected. Ambroxol also effectively inhibited enzymatic lipid peroxidation of microsomes. A 10 μM Fe^{3+}, 160 μM ADP and 100 μM NADPH-induced lipid peroxidation of microsomes was inhibited by ambroxol in a dose dependent fashion, and at 100 μM ambroxol, a 91.4% of inhibition was observed. The inhibitory effect of ambroxol on enzymatic lipid peroxidation was greater than that on nonenzymatic peroxidation.

To explore which reactive oxygen species are involved in the peroxidative action of Fe^{2+} and ascorbate on lipid, effects of oxidant scavengers on the damage were examined. As shown in Table I, the peroxidative action of 10 μM Fe^{2+} and 100 μM ascorbate on microsomal lipid components was inhibited by 30 μg/ml catalase, a scavenger of H_2O_2 and 10 mM DABCO, a quencher of $^1O_2$ but was not affected by 30 μg/ml superoxide dismutase (SOD), a scavenger of O_2 and 10 mM DMSO, a scavenger of OH · . Lipid peroxidation by 10 μM Fe^{3+}, 160 μM ADP and 100 μM NADPH was inhibited by 10 mM DABCO, while effects of 30 μg/ml SOD, 30 μg/ml catalase and 10 mM DMSO were not detected.

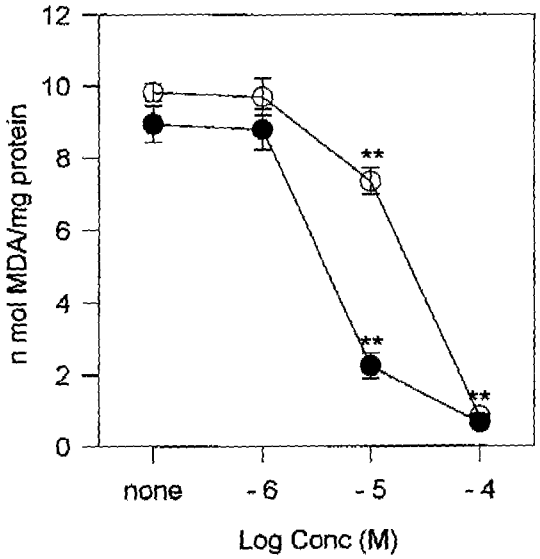

Fig. 1. Inhibition of iron and reducing agents-induced lipid peroxidation by ambroxol. Liver microsomes (0.2 mg protein/ml) were incubated with either 10 μM Fe^{2+} and 100 μM ascorbate (●) or 10 μM Fe^{3+}, 160 μM ADP and 100 μM NADPH (○). Values are means±SD, n=5. **<0.01 by Student's *t*-test.

Table I. Effects of oxidant scavengers on lipid peroxidation

Compounds	Fe^{2+}, ascorbate	Fe^{3+}, ADP, NADPH
	MDA production (n mol/mg protein)	
No addition	9.09±0.27	9.97±0.38
SOD 30 μg/ml	8.33±0.65	9.82±0.16
Catalase 30 μg/ml	7.58±0.30**	9.56±0.15
DMSO 10 mM	9.20±0.21	9.80±0.13
DABCO 10 mM	4.38±0.40**	8.00±0.18**

Liver microsomes (0.2 mg protein/ml) were incubated with either 10 μM Fe^{2+} and 100 μM ascorbate or 10 μM Fe^{3+}, 160 μM ADP and 100 μM NADPH. Values are means±SD, n=5. **p <0.01 by Student's *t*-test.

Inhibition of Fe^{2+}, H_2O_2 and Ascorbate-Induced De-

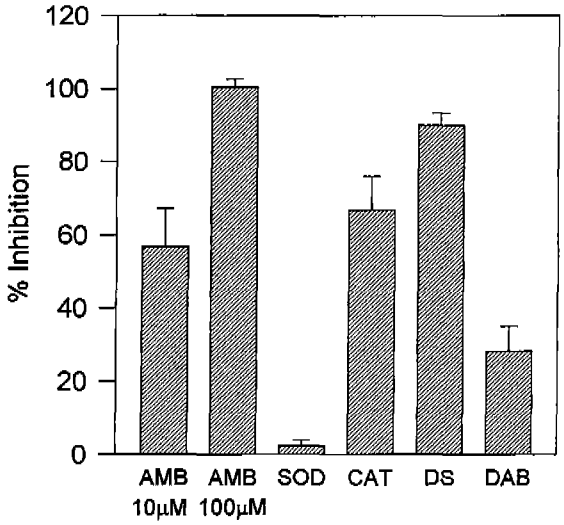

Fig. 2. Protective effect of ambroxol on degradation of hyaluronic acid by Fe^{2+}, H_2O_2 and ascorbate. Hyaluronic acid (1 mg/ml) was incubated with 10 μM Fc^{2+}, 500 μM H_2O_2 and 100 μM ascorbate for 1 h. Values are expressed as % inhibition and are means±SD, n=4. AMB, ambroxol; SOD, 30 μg/ml SOD; CAT, 30 μg/ml catalase; DS, 10 mM DMSO; DAB, 10 mM DABCO.

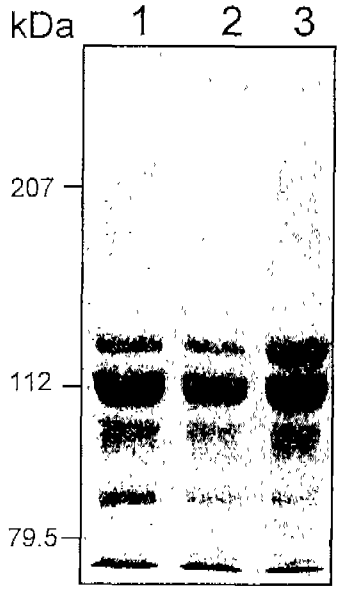

Fig. 3. Inhibition of the oxidative degradation of cartilage collagen by ambroxol. Cartilage collagen (200 μg/100μl) was incubated with 30 μM Fe^{2+}, 500 μM H_2O_2 and 200 μM ascorbic acid. Lane 1, intact collagen, and lane 2, collagen without ambroxol and lane 3, collagen+100 μM ambroxol in the presence of Fe^{2+}, H_2O_2 and ascorbate. Reference proteins were myosin (Mr, 207,000), β-galactosidase (Mr, 112,000) and bovine serum albumin (Mr, 79,500).

gradation of Hyaluronic Acid by Ambroxol

The oxidants react with hyaluronic acid and cause depolymerization, and then decrease of viscosity occurs (Greenward and Moy, 1980). The Protective action of ambroxol on the oxidative degradation of hyaluronic acid was examined. Hyaluronic acid (1 mg/ml) was incubated with 10 μM Fe^{2+}, 500 μM H_2O_2 and 100 μM ascorbate in the presence of ambroxol or not for 1 h. Viscosity of intact hyaluronic acid was 29.0±0.5 sec (means±SD, n=4). Designated concentrations of Fe^{2+}, H_2O_2 and ascorbate had a decreased viscosity of 7.7±0.5 sec, n=4. After 1 h of incubation, degradation of hyaluronic acid by 10 μM Fe^{2+}, 500 μM H_2O_2 and 100 μM ascorbate was completely inhibited by 100 μM ambroxol. The degradative action of Fe^{2+}, H_2O_2 and ascorbate on hyaluronic acid was inhibited by 30 μg/ml catalase, 10 mM DMSO and 10 mM DABCO, and 66.7%, 89.9% and 28.3% of inhibition was observed, respectively (Fig. 2). However, the inhibitory effect of 30 μg/ml SOD was not detected.

Antioxidant Action of Ambroxol on Collagen Degradation by Oxidants

The oxidative degradation of cartilage collagen was observed by electrophoretic analysis. Fig. 3 shows that when cartilage collagen (200 μg/100 μl) was treated with 30 μM Fe^{2+}, 500 μM H_2O_2 and 200 μM ascorbate at 37 °C for 6 h, a cleavage of major band (approximate Mr, 110,000) was detected. On the other hand, in this reaction cleavages of other bands were not observed. In contrast to lipid and hyaluronic acid, the degradative effect of Fe^{2+}, H_2O_2 and ascorbate on collagen was weak. The peroxidative action of Fe^{2+}, H_2O_2 and ascorbate on cartilage collagen was inhibited by 100 μM ambroxol.

Scavenging Effect of Ambroxol on Reactive Oxygen Species

The scavenging effect of ambroxol on superoxide anion was measured with reduction of ferricytochrome c by xanthine and xanthine oxidase. As can be seen in Table II, a 100 μM ambroxol did not affect reduction of cytochrome c by 0.2 mM xanthine and 10.5 mU/ml xan-

Table II. Scavenging action of ambroxol on superoxide anion

Compounds	Superoxide production (n mol/min)
No addition	7.03±0.03
SOD 30 μg/ml	0.48±0.06
Ambroxol 100 μM	7.03±0.02

Reduction of ferricytochrome c (75 μM) by 0.2 mM xanthine and 10.5 mU/ml xanthine oxidase was measured. Values are means±SD, n=3.

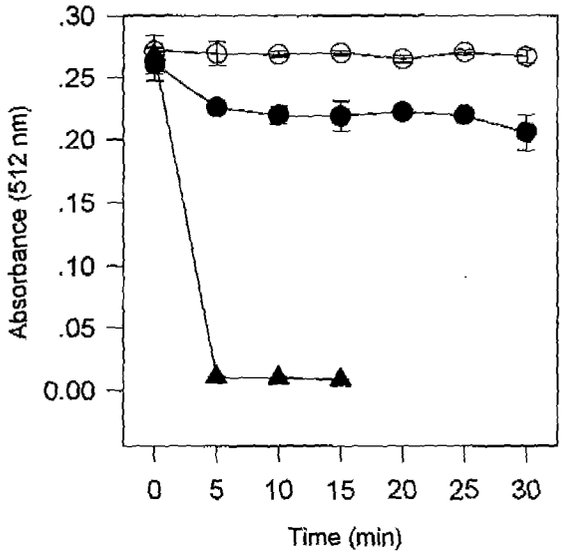

Fig. 4. Decomposing action of ambroxol on hydrogen peroxide. A 100 μM H_2O_2 was incubated with 100 μM ambroxol (●) (or 10 μg/ml catalase (▲) or not (○)) for the stated times. Values are means±SD of absorbance, n=4-7.

thine oxidase, which is responsible for SOD, while at this concentration, inhibitory effect of ambroxol on xanthine oxidase activity was not detected (data not shown).

Hydrogen peroxide has been implicated as a precursor for more reactive oxygen species (Kellogg III and Fridovich, 1977). The decomposing action of ambroxol on H_2O_2 was studied. H_2O_2 was completely decomposed by adding 10 μg/ml catalase within 5 min. Fig. 4 shows that ambroxol decomposed H_2O_2 with increasing incubation times, and at 30 min of incubation, concentration of H_2O_2 was decreased by 21.3%.

The scavenging effect of ambroxol on hydroxyl radicals produced was assayed by degradation of 2-α deoxyribose. Degradation of 2-α deoxyribose by either 50 μM Fe^{3+}, 50 μM EDTA, 500 μM H_2O_2 and 100 μM ascorbate or 0.2 mM xanthine and 42.1 mU/ml xanthine oxidase was inhibited by hydroxyl radical scavengers, 10 mM sodium formate and 10 mM DMSO. The xanthine and xanthine oxidase-induced degradation of 2-α deoxyribose was also inhibited by 30 μg/ml of SOD and catalase. As shown in Table III, a 100 μM ambroxol inhibited degradation of 2-α deoxyribose by either 50 μM Fe^{3+}, 50 μM EDTA, 500 μM H_2O_2 and 100 μM ascorbate or 0.2 mM xanthine and 42.1 mU/ml xanthine oxidase by 30.4-32.2%.

Compounds, which have antioxidant ability, are known to reduce free radical DPPH to 1,1-diphenyl-2-picrylhydrazine. Table IV shows that free radical DPPH was reduced by the addition of 1 mM cysteine, a scavenger, and 100 μM ambroxol, and reduction abilities of cysteine and ambroxol were prominent in initial phase (data not shown).

Table III. Scavenging action of ambroxol on hydroxyl radical

Compounds	Deoxyribose degradation	
	Fe^{3+}, EDTA, H_2O_2, Ascorbate	X./X.O.
No addition	0.986±0.019	99.0±9.7
Ambroxol 100 μM	0.686±0.015**	67.1±5.0**
SOD 30 μg/ml	-	69.2±7.7**
Catalase 30 μg/ml	-	57.8±8.5**
Sodium formate 10 mM	0.191±0.006**	78.5±3.2*
DMSO 10 mM	0.094±0.004**	73.3±6.8*

2-α Deoxyribose (2 mM) was treated with either 50 μM Fe^{3+}, 500 μM H_2O_2 and 100 μM ascorbate (expressed as Δabsorbance) or 0.2 mM xanthine and 42.1 mU/ml xanthine oxidase (expressed as fluorescence unit). Values are means±SD, n=5. *$p<0.05$, **$p<0.01$ by Student's *t*-test.

Table IV. Ambroxol effect on reduction of free radical DPPH

Compounds	Reduction of free radical DPPH (Δabsorbance/2 min)
No addition	0.028±0.001
Cysteine 1 mM	0.041±0.001
Ambroxol 100 μM	0.038±0.001

The reduction of DPPH (60 μM) was initiated by adding 100 μM ambroxol or 1 mM cysteine. Values are means±SD, n=3.

DISCUSSION

The oxidants released from activated phagocytic cells are thought to be implicated in the damage of the joint components associated with inflammation (Merry *et al.*, 1989; Weiss, 1989). In the inflamed sites, iron released from iron proteins in the synovial membranes appears to catalyze the formation of reactive oxygen species, such as OH · and iron-oxygen complexes, and cause the oxidative damages to tissue components, including cartilage and hyaluronic acid (Biemond *et al.*, 1986). Iron in the presence of reducing agents significantly degraded lipids of microsomes, hyaluronic acid and cartilage collagen, and which was inhibited by oxidant scavengers.

Ambroxol, a bromhexine metabolite, stimulates the release of surfactant by type II pneumocyte and is used as an expectorant. Ambroxol seems to have antiinflammatory property and antioxidant action. Ambroxol appears to inhibit chemotaxis of neutrophils and production

of interleukin 1 and tumor necrosis factor in monocytes and exert antiinflammatory action (Stockley *et al.*, 1988; Bianchi *et al.*, 1990). Both lipid peroxidation of lung tissues by heat or H_2O_2 and nonenzymatic peroxidation of linoleic acid are inhibited by ambroxol (Nowak *et al.*, 1994a and 1994b). In addition, ambroxol is found to scavenge OH · and HOCl (Lapenna *et al.*, 1994). However, ambroxol does not inhibit lung damage by hyperoxia (Nemery *et al.*, 1992) and is not able to protect liver lipids against oxidants (Nowak *et al.*, 1994b). Thus, at various tissues and under exposure of multiple oxidizing agents, antioxidant ability of ambroxol may be differently exerted.

The lipid peroxidation of liver microsomes caused by either Fe^{2+} and ascorbate or Fe^{3+}, ADP and NADPH was significantly inhibited by ambroxol in a dose dependent fashion. The peroxidative action of Fe^{2+} and ascorbate was inhibited by catalase and DABCO, while that of Fe^{3+}, ADP and NADPH was inhibited by DABCO but not by other scavengers. The character of enzymatic peroxidation of lipids may be somewhat different from nonenzymatic peroxidation. In the reaction, the peroxidative actions of both systems on lipids were not inhibited by DMSO, a scavenger of OH · . These findings support that iron-oxygen complexes are involved in iron-induced lipid peroxidation rather than reactive oxygen species (Halliwell and Gutteridge 1989b). Ambroxol may act as an effective antioxidant against lipid damage caused by iron-oxygen complexes. Antioxidant effect of ambroxol on the oxidants-induced degradation of hyaluronic acid and collagen was studied. Exposure of hyaluronic acid to Fe^{2+}, H_2O_2 and ascorbate caused depolymerized, and viscosity was decreased. In contrast to microsomal lipids, oxidants-induced degradation of hyaluronic acid was inhibited by DMSO, a scavenger of OH · , and the inhibitory effect of DMSO was greater than those of other scavengers. Thus, hydroxyl radical is considered to act as a strong oxidant in degradation of hyaluronic acid. And it is suggested that tissue components against the oxidants exhibit different responses according to their properties. Ambroxol (100 μM) almost completely inhibited degradation of hyaluronic acid by Fe^{2+}, H_2O_2 and ascorbate. Compare to lipid and hyaluronic acid, degradation of cartilage collagen by Fe^{2+}, H_2O_2 and ascorbate was weak. Ambroxol prevented cleavage of major band of collagen by Fe^{2+}, H_2O_2 and ascorbate. Ambroxol may exhibit antioxidant action on degradation of hyaluronic acid and collagen by its scavenging action on reactive oxygen species.

The scavenging action of ambroxol on reactive oxygen species produced was examined. Superoxide anion is generally accepted as less reactive oxygen species to initiate tissue damage, including lipid peroxidation and is postulated to be the precursor of more reactive oxygen species. Xanthine and xanthine oxidase system could not only easily produce reactive oxygen species but also regulate oxygen metabolism (Kellogg III and Fridovich, 1977; Greenward and Moy, 1979). In the superoxide producing xanthine and xanthine oxidase, the scavenging effect of ambroxol was not detected. This finding is not coincided with recent data that show scavening effect of ambroxol on O_2^- by 14.3±6.7% (Gillissen et al., 1997). However, we think that value does not have a significant meanning. H_2O_2 acts as a precursor for more reactive oxygen species (Kellogg III and Fridovich, 1977) and can also from complexes with iron (Halliwell and Gutteridge, 1989). As previous report (Nowak *et al.*, 1994a), ambroxol decomposed directly H_2O_2. The decomposing action of ambroxol on H_2O_2 was also supported by its inhibitory effect on degradation of hyaluronic acid by H_2O_2 (data not shown). Highly reactive OH · is known to damage directly most types of cellular macromolecules. This radical can be detected sensitively by measuring TBA reactivity of 2-α deoxyribose. Degradation of 2-α deoxyribose by either Fe^{3+}, EDTA, H_2O_2 and ascorbate or xanthine and xanthine oxidase differently responded to sodium formate and DMSO. In contrast to Fe^{3+}, EDTA, H_2O_2 and ascorbate system, the degradation of 2-α deoxyribose by xanthine and xanthine oxidase was less affected by OH · scavengers. Ambroxol significantly depressed degradation of 2-α deoxyribose by both systems. The results suggest that ambroxol has a strong scavenging action on OH · . However, the protective effect of ambroxol on tissue components seems to be not attributed to its chelating action on iron, because ambroxol does not affect autooxidation of Fe^{2+} (data not shown). The reduction of DPPH to 1,1-diphenyl-2-picrylhydrazine is used to test antioxidant ability of compounds, and its action can be able to compare with known antioxidants of free radical DPPH, such as cysteine and α-tocopherol (Constantin *et al.*, 1990; Lapenna *et al.*, 1992). Antioxidant ability of ambroxol can be supported by its reducing action on DPPH.

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