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Depressant Effects of Ambroxol and Erdosteine on Cytokine Synthesis, Granule Enzyme Release, and Free Radical Production in Rat Alveolar Macrophages Activated by Lipopolysaccharide

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Abstract: The present study examined the effects of ambroxol and erdosteine, bronchial expectorants, on the cytokine synthesis, granule enzyme release, and free radical production in rat alveolar macrophages activated by lipopolysaccharide. Ambroxol and erdosteine significantly decreased the production of tumour necrosis factors-α, interleukin-1β, and interleukin-6 in alveolar macrophages activated by lipopolysaccharide. These drugs significantly reduced the production of superoxide anion, hydrogen peroxide, and nitric oxide and the release of acid phosphatase and lysozyme in lipopolysaccharide-activated macrophages. Ambroxol and erdosteine showed no scavenging effect on superoxide anion and hydrogen peroxide, whereas both drugs effectively decomposed nitric oxide. The results show that ambroxol and erdosteine may inhibit the responses, including cytokine synthesis and free radical production, in rat alveolar macrophages activated by lipopolysaccharide. Unlike the production of reactive oxygen species, the inhibitory effect of ambroxol and erdosteine on the production of nitric oxide in lipopolysaccharide-activated alveolar macrophages may be accomplished by a scavenging action on the species and inhibition of the respiratory burst.

Alveolar macrophages are considered to play a central role in the regulation of immune response to inhaled pathogens and the development of pulmonary inflammation and fibrosis (Laskin & Pendino 1995). Interaction of macrophages with antigens leads to cellular activation, such as cytokine production (Nathan 1987). Tumour necrosis factor-α and interleukin-1β are known to mediate inflammatory events induced by lipopolysaccharide (LPS) in various tissues (Laskin & Pendino 1995; Schletter *et al.* 1995). Reactive oxygen and nitrogen species released by activated macrophages may play a part in tissue damage and organ disorders (Gunawardhana *et al.* 1993; Kiechle & Malinski 1993; Laskin *et al.* 1998). Reactive oxygen species and peroxynitrite are found to induce damage of lipids, proteins, and DNA and to oxidize protein and non-protein thiols (Gatti *et al.* 1994; Pryor *et al.* 1994; Halliwell & Gutteridge 1999).

Ambroxol (trans-4[(2-amino-3,5-dibromobenzyl)amino]-cyclohexanol hydrochloride) is known to promote bronchial secretion and is used as a bronchial expectorant (Disse 1987). Ambroxol has been shown to improve the clinical course of respiratory distress syndrome, including bronchopulmonary dysplasia (Wauer *et al.* 1992). Ambroxol inhibits the lipid peroxidation of lung tissue induced by hydrogen peroxide (Nowak *et al.* 1994) and reduces the hypochlorous acid-induced inactivation of α_1-antiproteinase (Cho *et al.* 1999). Ambroxol depresses functional responses of phagocytic cells stimulated by LPS or degraded immunoglobulin G (Lee *et al.* 1999; Park *et al.* 1999) and decreases the stimulated functional responses and cell death in rat alveolar macrophages exposed to silica (Kim *et al.* 2002).

Erdosteine, (±)-[[[(tetrahydro-2-oxo-3-thienyl)carbamoyl]-methyl]thio]acetic acid, is also used as a bronchial expectorants (Dechant & Noble 1996). Erdosteine and its metabolites are reported to inhibit the lipopolysaccharide-induced neutrophil influx into lung tissues, to decrease the production of reactive oxygen species in phagocytic cells activated by phorbol 12-myristate 13-acetate, and to protect lung tissues against hypochlorous acid, a reactive oxidant (Miyake *et al.* 1999; Hayashi *et al.* 2000).

The compounds that exert an antioxidant and augment the cellular antioxidant defense system may be of therapeutic use in some inflammatory diseases. Ambroxol and erdosteine are suggested to have an antioxidant effect. However, the effect of the compounds on stimulated responses in activated alveolar macrophages has not been clearly elucidated. Therefore, we investigated the effects of ambroxol and erdosteine on the cytokine synthesis, granule enzyme release, and free radical production in rat alveolar macrophages activated by lipopolysaccharide.

Materials and Methods

Materials. Ambroxol, lipopolysaccharide (from *E. coli*, LPS), superoxide dismutase (from bovine erythrocytes, SOD), catalase (from bovine liver), ferricytochrome c, hypoxanthine, xanthine oxidase (from buttermilk), phenol red, horseradish peroxidase, nitrate re-

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ductase (from *E. coli*), N^G-monomethyl-L-arginine (NMMA), NADPH, flavin adenine dinucleotide (FAD), sulfanilamide, N-(1-naphthyl)ethylenediamine dihydrochloride, S-nitroso-N-acetyl-D,L-penicillamine (SNAP), assay kit for acid phosphatase, phenolphthalein-glucuronic acid, *Micrococcus lysodeikticus*, dimethyl sulfoxide (DMSO), Hanks' balanced salt solution (HBSS), and Dulbecco's modified Eagle's medium (DMEM) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, U.S.A.). Erdosteine prepared under license from Edmond Pharma (Milan, Italy) was obtained from Daewoong Pharma (Seoul, South Korea). The enzyme-linked immunosorbent assay (ELISA) kits for interleukin-1β, interleukin-6, and tumour necrosis factor-α (TNF-α) were bought from Amersham Pharmacia Biotech UK Ltd. (Little Chalfont, Buckinghamshire, England). All other reagents were of analytical grade.

Animals were cared according to the NIH guidelines and the regulations of our university. Rats were maintained under a 12 hr light and dark cycle in a temperature-regulated (23±1°) animal room with water and food continuously available.

Isolation of rat alveolar macrophages. Sprague–Dawley rats weighing between 230 and 270 g each were anaesthetized by intraperitoneal injection of 40 mg/kg sodium pentobarbital. A tracheal cannula was inserted through an incision in the neck, and 6 ml of cold Ca^{2+}, Mg^{2+}-free phosphate buffered saline (PBS), pH 7.4, was instilled in the lung via a syringe attached to the cannula. Instillation of PBS was repeated three times to obtain macrophages. Cell suspensions were treated with hypotonic solution for the lysis of erythrocytes. The cell pellets suspended in Ca^{2+}, Mg^{2+}-free HBSS were placed on a Ficoll–Hypaque gradient and were centrifuged at 400×g for 45 min. at 4°. Macrophages were collected from the interphase of the gradient. The cells were washed with HBSS and were suspended in the same solution (Kim *et al.* 2002).

Measurement of cytokine production. Alveolar macrophages (3×10^5 cells/200 μl of mixtures) were treated with lipopolysaccharide (LPS) (1 μg/ml) for 18 hr at 37°. The amounts of cytokines produced were determined by the experimental procedures described in the assay kits for tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and interleukin-6 of Amersham Pharmacia Biotech. Cytokine concentrations were calculated from the standard curve by using known amounts of recombinant cytokines (rIL-6, rIL-1β, and rTNF-α from Amersham Pharmacia Biotech) and were expressed as picograms/ml.

Measurement of superoxide anion production. The superoxide anion produced was assayed by superoxide dismutase-inhibitable reduction of ferricytochrome c (Kim *et al.* 2001). The reaction mixtures (200 μl) in 96 well microplate contained 3×10^5 macrophages, 75 μM ferricytochrome c, 1 μg/ml LPS, and DMEM, pH 7.4 and were placed at 5% CO_2 incubator, 37° for 6 hr. Absorbance was measured using a microplate reader (Spectra MAX 340; Molecular Devices, Co., Sunnyvale, CA, U.S.A.). The amount of reduced ferricytochrome c was expressed as nanomolar concentration by using the molar extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} at 550 nm.

Measurement of hydrogen peroxide production. Macrophages (3×10^5 cells) were incubated in 200 μl of DMEM containing 1 μg/ml LPS, 0.1 mg/ml phenol red, and 0.2 mg/ml horseradish peroxidase for 6 hr at 37°. The reaction was terminated by adding 20 μl of 1 N NaOH, and absorbance was measured at 610 nm (Kim *et al.* 2001). The concentration of hydrogen peroxide was calculated using hydrogen peroxide solution as the standard.

Measurement of nitrite/nitrate production. Nitric oxide production by the nitric oxide synthase in macrophages was measured by assaying nitric oxide metabolites, nitrite and nitrate (NO_X) (Gilad *et al.* 1998; Kim *et al.* 2001). Macrophages (3×10^5 cells/200 μl) were incubated in DMEM containing 1 μg/ml LPS for 6 hr at 37°. Nitrate in the culture medium was reduced to nitrite by incubation with nitrate reductase (500 munits/ml), 160 μM NADPH, and 4 μM FAD at room temperature for 2 hr. The medium was mixed with equal amount of Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride, and 2.5% phosphoric acid). Absorbance was measured at 550 nm, and the amount of nitrite produced was determined using sodium nitrite as the standard. The results were expressed as total nitrite equivalents (NO_X).

Measurement of acid phosphatase release. Macrophages (3×10^5) were incubated in 200 μl of DMEM containing 1 μg/ml LPS for 18 hr at 37°. Acid phosphatase released was measured as a hydrolysis of *p*-nitrophenyl phosphate by using diagnostic kit for the enzyme (Sigma-Aldrich Inc.). Absorbance was measured at 405 nm. The activity of acid phosphatase was estimated from the standard curve by using a *p*-nitrophenol standard reagent and was expressed as munits/3×10^5 cells.

Measurement of lysozyme release. The release of lysozyme from macrophages was measured using *Micrococcus lysodeikticus* suspension as described in the method of Kim *et al.* (2001). Macrophages (3×10^5) were suspended in 200 μl of DMEM containing 1 μg/ml LPS and were incubated for 18 hr at 37°. Forty microliters of *Micrococcus* suspension (50 mg *Micrococcus lysodeikticus* in 5 ml of 0.65 M potassium phosphate buffer, pH 6.2) was added to the mixtures. After a 30 min. incubation at 37°, adding 50 μl of 1 N acetic acid and stopped the incubation. Absorbance was measured at 450 nm.

Assay of scavenging action on reactive species. The decomposing effect of ambroxol or erdosteine on superoxide anion was measured by using a reduction of ferricytochrome c (Trayor & Mayeux 1997). The conversion of hypoxanthine to urate by xanthine oxidase liberates superoxide anion, which induces a reduction of ferricytochrome c. The reaction mixtures contained 75 μM ferricytochrome c, 0.2 mM hypoxanthine, 10.5 munits/ml xanthine oxidase, 150 mM KCl, 50 mM NaH_2PO_4 buffer, pH 7.4 and ambroxol (or erdosteine).

The decomposing effect on hydrogen peroxide was measured in the mixtures contained 20 μM H_2O_2, 0.1 mg/ml phenol red, 0.1 mg/mlhorseradish peroxidase, 150 mM KCl, 50 mM NaH_2PO_4 buffer, pH 7.4, and ambroxol (or erdosteine). The reaction was performed for 15 min. at 30°. The hydrogen peroxide remaining was measured and calculated as previously described (Piedad *et al.* 1997).

The decomposing effect of ambroxol on nitric oxide generated from SNAP was determined by measuring nitrite produced (Janusz 1997). The reaction mixture contained 100 μM SNAP, 120 mM KCl, 50 mM NaH_2PO_4 buffer, pH 7.4, and ambroxol (or erdosteine). After a 30 min. incubation at 30°, the mixtures were mixed with an equal amount of Griess reagent. Absorbance was measured at 550 nm, and amount of nitrite remained was determined using sodium nitrite as the standard.

Data analysis. Data are expressed as mean±S.E.M. The data were analyzed by one-way analysis of variance. The analysis of variance justified post hoc comparison between the different groups by using the Duncan's test. A probability of $P<0.05$ was considered to be statistically significant.

Results

Inhibitory effect of ambroxol and erdosteine on lipopolysaccharide-induced cytokine production.

Macrophages exposed to stimulating agents effectively discharge a variety of proinflammatory cytokines such as tumour necrosis factor-α and interleukin-1β, which are involved in tissue injury. We investigated the effect of ambroxol or erdosteine on the production of tumour necrosis factor-α, interleukin-1β, and interleukin-6 in rat alveolar

macrophages activated by lipopolysaccharide. Alveolar macrophages treated with 1 μg/ml of lipopolysaccharide for 18 hr produced 105.0 pg of tumour necrosis factor-α/3×10^5 cells, 26.1 pg of interleukin-1β/3×10^5 cells, and 129.1 pg of interleukin-6/3×10^5 cells. Fig. 1 shows that ambroxol and erdosteine (1–100 μM) significantly decreased the lipopolysaccharide-induced production of cytokines in macrophages in a concentration-dependent manner. Ambroxol at 100 μM showed the 60, 41, and 35% of inhibitory effects on the production of tumour necrosis factor-α, interleukin-1β, and interleukin-6, respectively. The inhibitory effect of erdosteine on the cytokine production was less than that of ambroxol.

Effect of ambroxol and erdosteine on lipopolysaccharide-induced free radical production.

Effect of ambroxol or erdosteine on the respiratory burst stimulated by lipopolysaccharide in alveolar macrophages was examined. Intact macrophages liberated 1.34±0.1 nmol superoxide anion/3×10^5 cells and 1.06±0.20 nmol hydrogen peroxide/3×10^5 cells for 6 hr of incubation. Alveolar macrophages treated with 1 μg/ml of lipopolysaccharide for 6 hr released 5.02 nmol superoxide anion/3×10^5 cells and 3.31 nmol hydrogen peroxide/3×10^5 cells. Ambroxol or erdosteine (1–100 μM) attenuated the lipopolysaccharide-stimulated superoxide anion and hydrogen peroxide production in a concentration-dependent manner, and at 100 μM, the compounds decreased the production of reactive oxygen species by 25 to 76% (fig. 2). The depressant effect of ambroxol on production of reactive oxygen species was greater than that of erdosteine on the basis of concentration.

In the present study, nitric oxide produced was assayed by measuring nitric oxide metabolites (NO_X). Intact macrophages liberated 2.10±0.15 μM NO_X/3×10^5 cells for 6 hr of incubation. Alveolar macrophages stimulated with 1 μg/ml of lipopolysaccharide for 6 hr of incubation produced 5.72 μM NO_X/3×10^5 cells. The NO_X production in macrophages treated with lipopolysaccharide was significantly decreased by the addition of 500 μM NMMA, an inhibitor of nitric oxide synthase. Ambroxol or erdosteine significantly decreased the LPS-induced nitric oxide production in a concentration-dependent manner (fig. 3). Erdosteine showed an inhibitory effect on the nitric oxide production less than ambroxol.

Effect of ambroxol and erdosteine on granule enzymes release induced by lipopolysaccharide.

The secretion of granule enzyme by activated alveolar macrophages was assayed by measuring the release of acid phosphatase and lysozyme. Alveolar macrophages exposed to 1 μg/ml of lipopolysaccharide for 18 hr discharged acid phosphatase and lysozyme. Fig. 4 shows that when macrophages were incubated with ambroxol or erdosteine, the lipopolysaccharide-induced release of acid phosphatase and lysozyme was reduced with concentration of drug. Like the finding in the respiratory burst, the inhibitory effect of ambroxol was greater than that of erdosteine. Combined addition of ambroxol and erdosteine did not show a potentiating effect on the release of acid phosphatase.

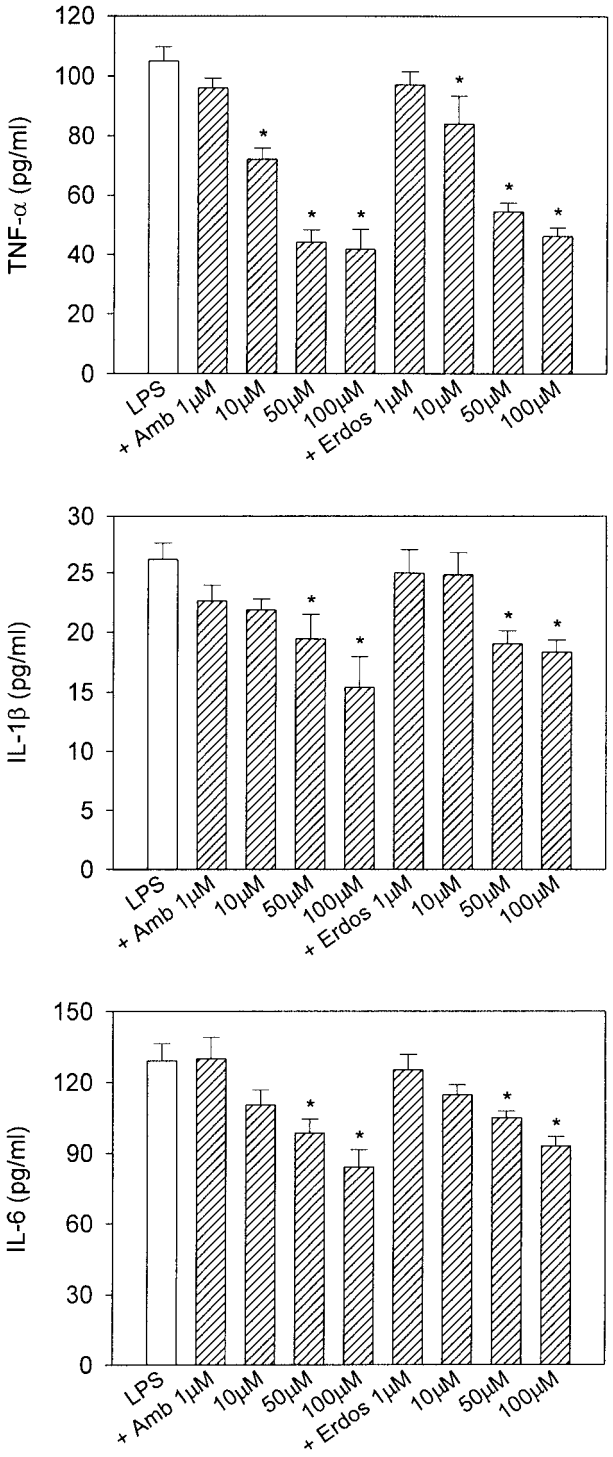

Fig. 1. Effect of ambroxol and erdosteine on lipopolysaccharide (LPS)-induced production of tumour necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6. Alveolar macrophages (3×10^5 cells/200 μl) were treated with 1 μg/ml of LPS in the presence of various concentrations of ambroxol and erdosteine for 18 hr. Data represent mean±S.E.M. (n=5). *$P<0.05$, significantly different from LPS.

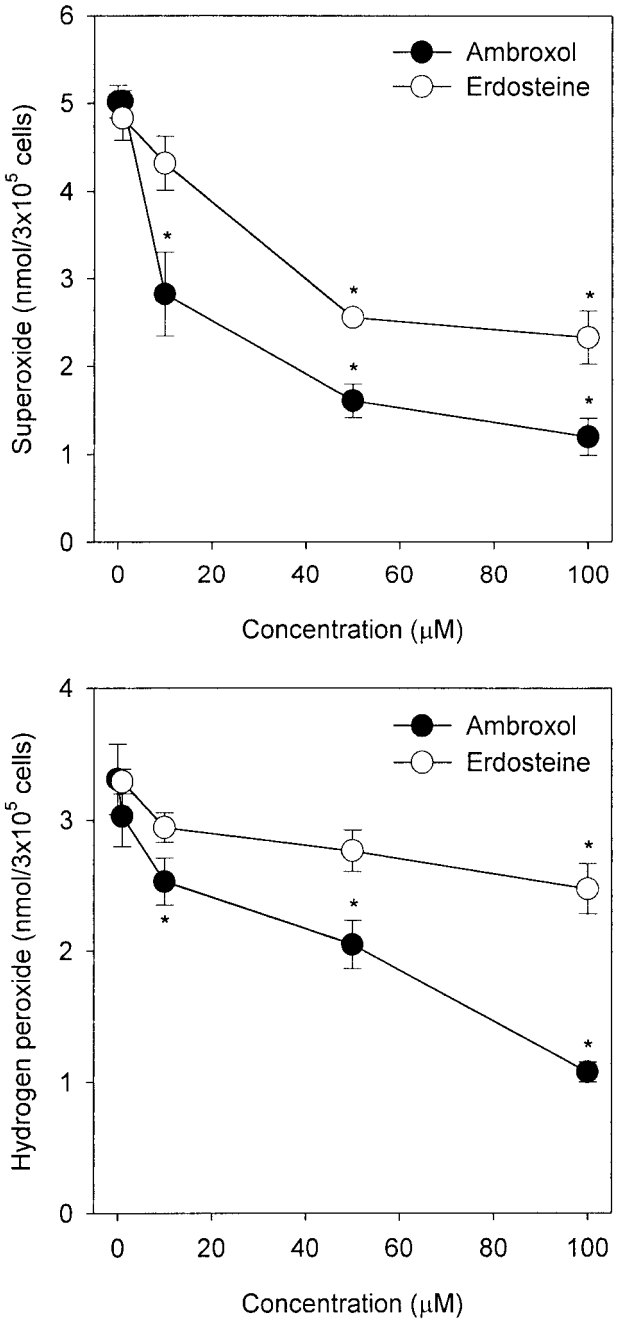

Fig. 2. Effect of ambroxol and erdosteine on LPS-induced production of superoxide and hydrogen peroxide. Alveolar macrophages (3×10^5 cells/200 μl) were incubated with 1 μg/ml of LPS in the presence of various concentrations of drugs for 6 hr. Data represent mean±S.E.M., n=5. *P<0.05, significantly different from no addition of drugs.

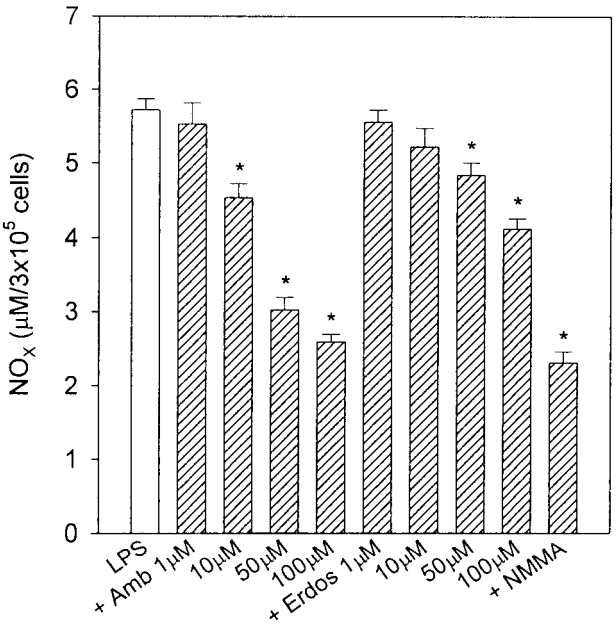

Fig. 3. Effect of ambroxol and erdosteine on production of nitric oxide in macrophages activated by LPS. Macrophages (3×10^5 cells/200 μl) were treated with 1 μg/ml of LPS in the presence of various concentrations of drugs or 500 μM NMMA for 6 hr. Data represent mean±S.E.M., n=5. *P<0.05, significantly different from LPS.

Scavenging effect of ambroxol and erdosteine on reactive species.

The present study examined whether effects of the compounds on the production of free radicals by macrophages may be attributed to the scavenging action on reactive species. The scavenging action of ambroxol and erdosteine on superoxide anion was measured by looking at the effect on the reduction of ferricytochrome c induced by superoxide anion formed by reaction of hypoxanthine with xanthine oxidase, which was significantly decreased by 10 μg/ml superoxide dismutase. As shown in fig. 5, ambroxol or erdosteine (10 and 100 μM each) did not decrease the reduction of ferricytochrome c induced by superoxide anion formed by reaction of 0.2 mM hypoxanthine with 10.5 munits/ml xanthine oxidase. The same concentrations of compounds alone did not reduce the activity of xanthine oxidase (data not shown).

Hydrogen peroxide is attained from the dismutation of the superoxide anion and is well known as a precursor of highly reactive species (Kim *et al.* 2001). We examined the decomposing effect of ambroxol and erdosteine on hydrogen peroxide. Catalase (10 μg/ml) significantly decomposed hydrogen peroxide, whereas ambroxol and erdosteine (10 and 100 μM each) did not show a decomposing effect on hydrogen peroxide (fig. 5).

The scavenging action of ambroxol or erdosteine on nitric oxide was measured by measuring the effect on nitrite liberated from SNAP. Ambroxol, erdosteine, and 1 mM GSH significantly decomposed the nitrite produced from SNAP (fig. 5). Ambroxol or erdosteine at 100 μM removed 70 and 48% of nitric oxide, respectively.

Discussion

A bacterial infection of the respiratory tract results in transient elevation of tumour necrosis factor-α, interleukin-1β,

and interleukin-6 concentration in the lung (Victor *et al.* 1999; Michel 2000). Inflammatory cytokines released from macrophages play an important role in the regulation of endotoxin-mediated tissue injury (Laskin & Pendino 1995). Ambroxol and erdosteine have been suggested to depress responses of activated phagocytic cells. Therefore, we investigated the effect of ambroxol or erdosteine on the production of tumour necrosis factor-α, interleukin-1β, and interleukin-6 in activated rat alveolar macrophages. Exposure of alveolar macrophages to lipopolysaccharide showed a significant increase in production of tumour necrosis factor-α, interleukin-1β, and interleukin-6. Depressant effect of the compounds on the cytokine synthesis in lipopolysaccharide-activated macrophages indicates that ambroxol or erdosteine may provide a beneficial effect in alleviation of the lung injury mediated by cytokines.

Reactive oxygen species appear to be implicated in tissue damage in various pathologic conditions. The destruction of bronchial tissue associated with inflammation is ascribed in part to the role of free radicals (Weiss 1989; Halliwell & Gutteridge 1999). We investigated the depressant mechanism of erdosteine on the respiratory burst of alveolar macrophages exposed to lipopolysaccharide. It has been

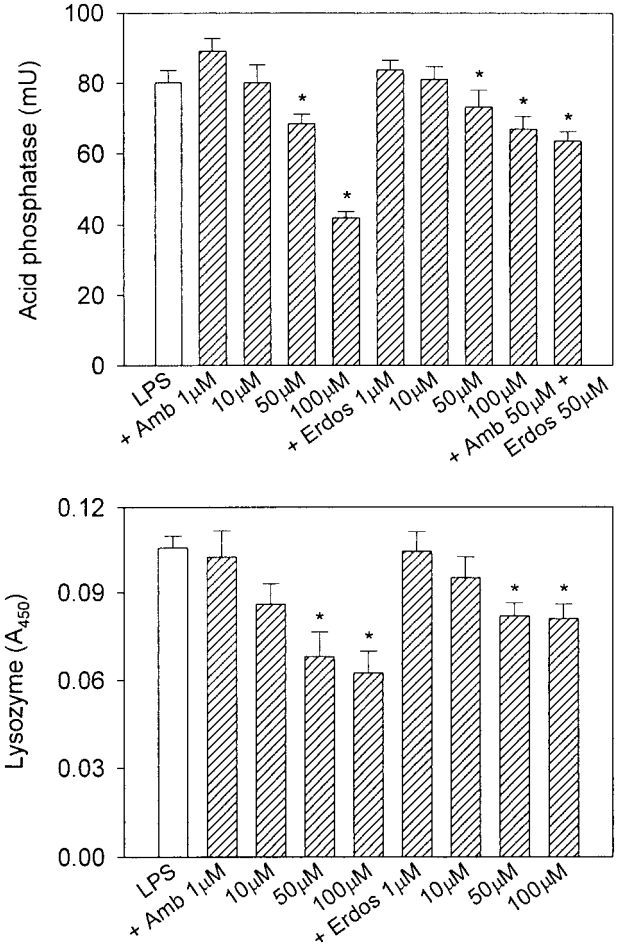

Fig. 4. Effect of ambroxol and erdosteine on LPS-induced release of granule enzymes. Alveolar macrophages (3×10^5 cells/200 μl) were treated with 1 μg/ml of LPS in the presence of various concentrations of drugs for 18 hr. Data represent mean±S.E.M., n=5. *P<0.05, significantly different from LPS.

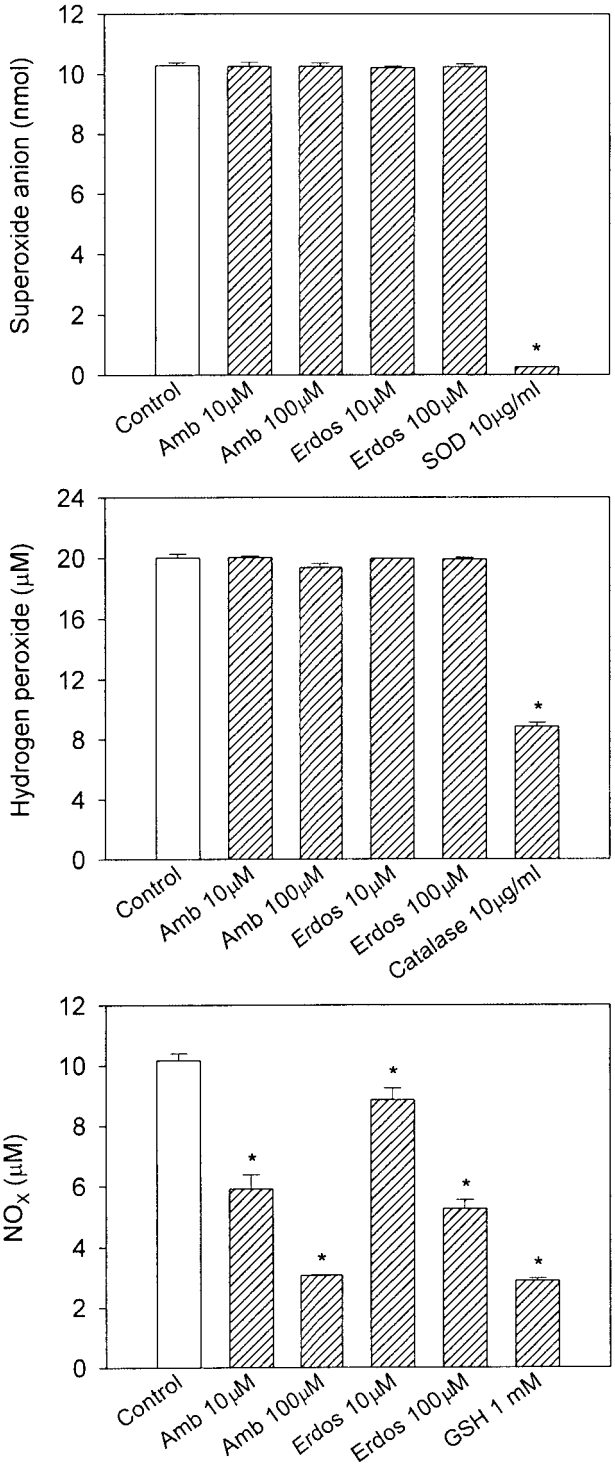

Fig. 5. Scavenging effects of ambroxol and erdosteine on reactive oxygen species and nitric oxide. Experimental conditions were the same as described in Materials and Methods. Data represent mean±S.E.M., n=5. *P<0.05, significantly different from the control.

suggested that the inhibitory effect of erdosteine on the respiratory burst in inflammatory cells in vivo may be accomplished by the metabolites containing sulfhydryl groups (Miyake *et al.* 1999; Braga *et al.* 2000). In contrast to these reports, we found that erdosteine, which does not contain sulfhydryl groups, decreased the production of reactive oxygen species and nitric oxide as well as cytokine production in lipopolysaccharide-activated macrophages. In addition, ambroxol without sulfhydryl groups also significantly inhibited production of the same reactive species, and the effect was greater than erdosteine. This finding indicates that erdosteine may reduce the respiratory burst of alveolar macrophages without intervention of the role of sulfhydryl groups. Ambroxol and erdosteine appear to exert a protective effect on lung tissues against free radicals by inhibition of the respiratory burst in phagocytic cells.

Phagocytic cells exposed to stimulating agents discharge granule enzymes that are involved in tissue injury associated with inflammation. Ambroxol has been shown to attenuate the stimulated respiratory burst and granule enzyme release in human neutrophils activated by degraded immunoglobulin G (Park *et al.* 1999). Lipopolysaccharide significantly stimulated the release of acid phosphatase and lysozyme from rat alveolar macrophages. Inhibitory effect of ambroxol on the production of free radicals as well as lysosomal enzyme release in rat alveolar macrophages activated by lipopolysaccharide was similar to results in the previous report using the same cells (Lee *et al.* 1999). Like the production of cytokines and reactive oxygen species, the inhibitory effect of ambroxol on release of lysosomal enzymes was greater than that of erdosteine. In the cell viability assay using MTT (3–4,5-dimethylthiazoyl-2-yl)-2,5-diphenyltetrazolium bromide), the ambroxol and erdosteine up to 100 μM did not show a cytotoxic effect on alveolar macrophages (data not shown). The present results indicate that the cytotoxic effect may not mediate depressant effects of ambroxol and erdosteine on functional responses of lipopolysaccharide-activated macrophages. Co-addition of ambroxol and erdosteine did not enhance their effect on the production of reactive oxygen species (data not shown) and release of granule enzyme. This finding suggests that both drugs appear to show an effect on macrophage responses through a similar action.

Ambroxol has been shown to scavenge hydroxyl radicals and hypochlorous acid (Gillissen & Nowak 1998), while the decomposing effects on superoxide anion and hydrogen peroxide are uncertain (Gillissen *et al.* 1997; Cho *et al.* 1999). Compared with the active metabolites, the scavenging effect of erdosteine on free radicals is not clear (Miyake *et al.* 1999; Braga *et al.* 2000). The results show that ambroxol and erdosteine do not have a decomposing effect on superoxide anion and hydrogen peroxide. Therefore, the depressant action of ambroxol and erdosteine on the production of reactive oxygen species in activated macrophages may be accomplished by inhibition of functional responses rather than the scavenging action. In contrast to production of reactive oxygen species, the effect of ambroxol or erdosteine on the production of nitric oxide may be partially ascribed to their scavenging action on the same species. The compounds, which have an antioxidant effect, could provide a protective effect on tissue components against oxidants (Whiteman *et al.* 1996; Kim *et al.* 1999). Co-addition of iron and ascorbate has been reported to stimulate cyclosporine-induced damage of mitochondria and epithelial cell line of kidney (Lee *et al.* 2001). The reaction of superoxide anion with nitric oxide produces a reactive oxidant peroxynitrite that is involved in the tissue destruction in inflammatory diseases (Beckman *et al.* 1994; Whiteman *et al.* 1996; Halliwell & Gutteridge 1999). Therefore, the scavenging effect of ambroxol and erdosteine on nitric oxide may provide a protective effect on lung tissues against the damaging action of peroxynitrite.

In conclusion, ambroxol and erdosteine may attenuate the functional responses of rat alveolar macrophages including cytokine synthesis. Ambroxol and erdosteine may show an inhibitory effect on free radical production in activated macrophages by a different action. Unlike the production of reactive oxygen species, the effect of compounds on the production of nitric oxide by activated macrophages may be partially accomplished by the scavenging action on the species.

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