Protective effect of ambroxol against heat- and hydrogen peroxide-induced damage to lung lipids in mice

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Protective effect of ambroxol against heat- and hydrogen peroxide-induced damage to lung lipids in mice. D. Nowak, A. Antczak, T. Pietras, P. Bialasiewicz, M. Krol. ©ERS Journals Ltd 1994.

ABSTRACT: We wanted to determine whether ambroxol, a drug which stimulates the release of surfactant by type II pneumocytes, can protect lung lipids from peroxidative damage in mice.

Animals were injected intraperitoneally with ambroxol, 0.169 mmol·kg^{-1}, or 1 ml buffer once a day for three consecutive days. Lipid peroxidation was then induced in lung homogenates either by means of heat, 50°C, or H_2O_2, 10 mmol·l^{-1}.

The lung homogenates from ambroxol-treated animals revealed decreased lipid peroxidation in response to both stimuli. The heat- and H_2O_2-induced generation of conjugated dienes (a first lipid peroxidation product) in ambroxol-treated lung homogenates was 3.7 and 3.1 fold lower than in the lungs from buffer-injected mice. Ambroxol, as an inhibitor of heat- and H_2O_2-induced lipid peroxidation, was equipotent to and stronger than the two antioxidants, N-acetylcysteine and methionine, respectively. Ambroxol was not able to protect heart and liver lipids.

These results suggest that ambroxol can sufficiently enhance the antioxidant defence in lung tissue and can act as a lung lipid antioxidant.
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Reactive oxygen species and free radical lipid peroxidation may initiate pathological changes leading to the development of lung diseases, such as pulmonary emphysema, adult respiratory distress syndrome and lung cancer [1, 2]. One possible method of preventing these diseases is the pharmacological enhancement of pulmonary antioxidant defence or suppression of free radical generation in lung tissue. The drug useful for this purpose should sufficiently scavenge reactive oxygen species and/or inhibit influx of activated phagocytes, which are the main source of reactive oxygen species in the respiratory tract.

Many studies have indicated that ambroxol (2-Amino-3,5-dibromo-N-[trans-4-hydroxycyclohexyl] benzylamine), a drug from the expectorant class which stimulates the formation and release of surfactant by type II pneumocytes [3], may be a putative inhibitor of free radical-mediated processes in lung tissue [4]. Ambroxol was found to protect α_1-proteinase inhibitor from oxidative inactivation [5], and to inhibit the chemotactic response and spontaneous migration of human polymorphonuclear leucocytes (PMNLs) [6, 7]. Ambroxol also attenuated the zymosan-induced chemiluminescence of human PMNLs and alveolar macrophages [8], and inhibited interleukin-1 and tumour necrosis factor production by human mononuclear cells stimulated with lipopolysaccharide [9]. This drug diminished the bleomycin-induced lung injury in rats [10], and decreased their mortality after administration of paraquat, a herbicide which generates reactive oxygen species [11]. Recently, we found that intraperitoneal administration of ambroxol protected lung and heart lipids from oxidative stress provoked by intravenous injection of endotoxin in mice [12]. In this animal model, ambroxol was found to be equipotent to N-acetylcysteine, a well-known antioxidant [13], in inhibition of lipid peroxidation [14]. Moreover, this drug was also able to scavenge hypochlorous acid and hydroxyl radicals (OH·), and to inhibit lipid peroxidation *in vitro*. At concentrations of 1–5 mM, it completely inhibited generation of malondialdehyde from linoleic acid incubated with an OH·-generating system (Fe^{2+} 10 μm, H_2O_2 280 μM, ethylene diamine tetra-acetic acid (EDTA) 20 μM) [15].

Taking the above into consideration, it seems that ambroxol may protect lung tissue, especially lung lipids, from peroxidative damage. Unfortunately, there is no basic information yet available on its capacity to act as lipid antioxidant *in vivo*. Therefore, the aim of the present study was to explore whether intraperitoneal administration of ambroxol changes the susceptibility of lung lipids to peroxidative damage. By measurement of conjugated dienes (CD), a first lipid peroxidation product, we have found that lung homogenates from ambroxol-treated animals revealed decreased lipid peroxidation in response to thermal and chemical stimuli. The protective effect of ambroxol on lung lipids was stronger than that caused by the two antioxidants, N-acetylcysteine and methionine.

Material and methods

Reagents

Ambroxol hydrochloride was a kind gift from W. Trautner (Boehringer Ingelheim, Vienna). N-acetyl-cysteine was from the Zambon Group (Switzerland). Methionine was obtained from Serva Feinbiochemica (Heidelberg). Chloroform (for spectroscopy), and heptane were from Ubichem (UK). All other reagents were purchased from POCH (Poland). Ambroxol, N-acetyl-cysteine and methionine were dissolved in 0.2 mol·l^{-1} phosphate buffer (pH 6.4) and filtered through 0.3 µm Millipore filter immediately before use.

Experimental protocol

Male Balb/c mice, 26–31 g and 5–7 weeks of age, were maintained at room temperature and allowed free access to food and water. The mice were injected intra-peritoneally with ambroxol (70 mg·kg^{-1}), N-acetylcysteine (27.6 mg·kg^{-1}), methionine (25.2 mg·kg^{-1}), or 1 ml buffer alone, once a day for three consecutive days. The molar doses of all drugs were equal (0.169 mmol·kg^{-1} of body weight). The animals were sacrificed by vertebral dislocation, 3 h after the last intraperitoneal injection. The thoracic cavity was opened and lungs, heart and liver were excised. Organs were immediately washed with ice-cold phosphate-buffered saline (PBS) (pH 7.4) and then homogenized (125 mg of wet organ in 1 ml PBS). The organ homogenates were stored at -80°C under nitrogen (for not more than for 7 days) until thermal or chemical induction of lipid peroxidation was performed.

Induction of lipid peroxidation

Thermal induction. Each defrosted individual organ homogenate was divided into two parts. One part was incubated at 50°C, and the second (control) was kept at 0°C. After 60 min incubation, the lipid peroxidation was immediately estimated by measurement of CD [16], as described previously [17]. Briefly, 1 ml of organ homogenate was mixed with 7 ml of chloroform-methanol (1:2 vol/vol), shaken for 2 min and centrifuged (15,00×g for 5 min). Five millilitres of the lower (chloroform) layer was mixed with 2 ml of distilled water acidified with 0.1 N HCl to pH 2.5. The mixture was again shaken for 2 min and centrifuged as above. The chloroform layer was aspirated and dried under a flow of nitrogen gas. The residue was reconstituted with 1 ml of heptane, and its absorbance was read against a heptane blank at 233 nm.

Chemical induction. H_2O_2 (final concentration 10 mmol·l^{-1}) was added to one portion of the organ homogenate. The control portion received the same amount of deionized water. Samples of lung, liver and heart homogenates were incubated at 37°C for 20, 15 and 20 min, respectively, and the content of CD was then measured.

Statistical analysis

Results are expressed as the mean±SD value of absor-bance readings at 233 nm·g^{-1} of wet organ. The indi-vidual increments in the content of CD were calculated by subtracting absorbance readings at 233 nm found for samples incubated at 0°C from values obtained at 50°C and by subtracting values obtained with deionized water from those observed when H_2O_2 was added. This pro-cedure was performed for all organ homogenates from mice injected with buffer, ambroxol, N-acetylcysteine or methionine, and the results were compared by anal-ysis of variance (ANOVA). The differences between results (the mean basal CD content, the mean CD content after treatment with H_2O_2 or heat, the mean increment in CD content) found in organ homogenates from control buffer-injected mice and those obtained for each drug-treated animal group were determined by multivariate analysis of variance (MANOVA). A p-value of less than 0.05 was considered to be significant.

Results

Heat- and H_2O_2-induced generation of CD in lung, heart and liver homogenates

Preliminary experiments were performed to find opti-mal conditions of chemical and thermal induction of lipid peroxidation in lung, liver and heart homogenates. The relationship between the increment in the content of CD and the temperature, time of incubation and the concentration of H_2O_2 was analyzed for all organ homo-genates. Figure 1 shows the effect of incubation time at both 0° and 50°C on the content of CD in lung and heart homogenates. After 30 min incubation, both organ homogenates revealed significant increase in the content of CD compared to the samples incubated at 0°C.
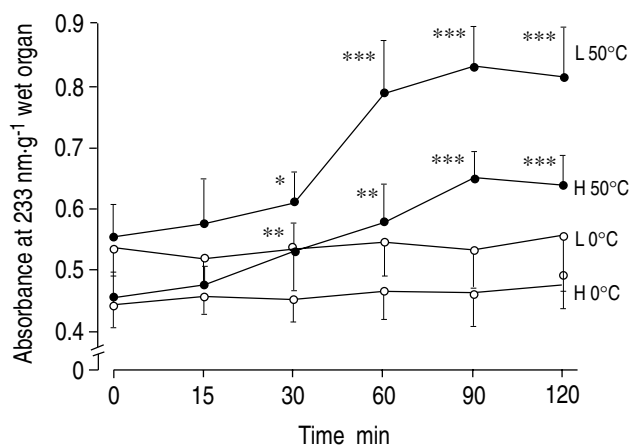

Fig. 1. – Effect of incubation time at 0°C (——○——) and 50°C (——●——) on the content of conjugated dienes (CDs) in lung (L) and heart (H) homogenates. Results, expressed as mean±SD of absorbance readings at 233 nm·g^{-1} of wet organ, were obtained from seven separate experiments with organs from seven mice. Significance of difference from the equivalent value obtained at 0°C: *: p<0.05; **: p<0.01; ***: p<0.001.
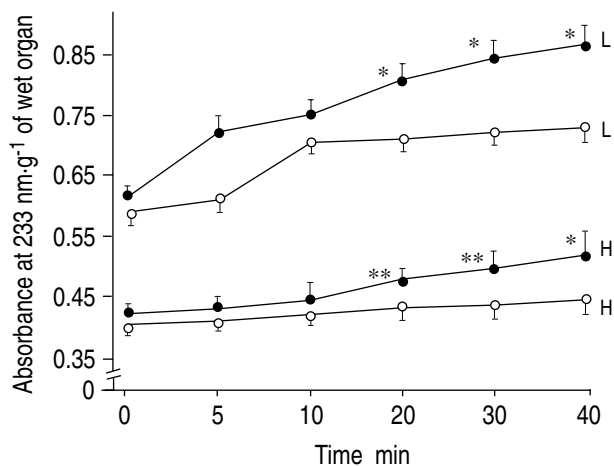
Fig. 2. – Effect of incubation time at 37°C on the H_2O_2-induced increase in the content of conjugated dienes (CDs) in lung (L) and heart (H) homogenates. Results, expressed as mean±SD of absorbance readings at 233 nm·g⁻¹ wet organ, were obtained from three separate experiments with organs from three mice. Significantly different from the appropriate value obtained in the presence of deionized water: *: $p < 0.05$; **: $p < 0.01$. —●—: incubation with 10 mmol·l^{-1} H_2O_2;—○—: control incubation with deionized water.

Similar results were obtained for liver homogenates (data not shown).

Incubation of lung and heart homogenates with 10 mmol·l^{-1} H_2O_2 for ≥20 min resulted in a significant increase in the content of CD (fig. 2). Under these conditions, 2 and 5 mmol·l^{-1} H_2O_2 had no significant effect on CD formation (data not shown). Similar results were obtained for liver homogenates; a significant increase in the content of CD was already observed after 15 min incubation (data not shown).

Based on these results, 60 min incubation at 50°C was used in further experiments for thermal induction of lipid peroxidation in all organ homogenates. 20 min incubation with 10 mmol·l^{-1} H_2O_2 was chosen for chemical induction of lipid peroxidation in lung and heart homogenates. Only liver homogenates were incubated for 15 min.

Effect of drug treatment on the basal CD content in lungs and other organs

The effect of ambroxol, N-acetylcysteine and methionine on the basal CD levels in selected organs in mice was examined in two series of experiments. All drugs injected intraperitoneally for three consecutive days influenced the CD levels in lung, heart and liver homogenates (figs 3 and 4). In the first series of experiments, ambroxol significantly decreased the CD content in the murine heart and liver, by 1.5 ($p < 0.01$) and 1.3 fold ($p < 0.001$), respectively, (fig 3b and c). Lungs from ambroxol-injected mice revealed only a tendency to have lower CD content compared to buffer-treated animals (fig. 3a). However, in the second series of experiments, ambroxol significantly decreased the CD level in all organ homogenates (fig. 4). The absorbance readings at 233 nm·g⁻¹ of wet organ for lungs, heart and liver from the ambroxol group were, respectively, approximately 1.1 ($p < 0.02$), 1.2 ($p < 0.01$) and 1.1 fold ($p < 0.01$) lower than corresponding values observed in buffer-injected mice.

N-acetylcysteine and methionine significantly lowered or tended to decrease the pulmonary and liver basal CD content in both sets of experiments (fig. 3a and c, and fig. 4a and c). The influence of N-acetylcysteine and methionine on the heart content of CD was variable being either a decrease (fig. 3b) or increase (fig. 4b), although not always significant.
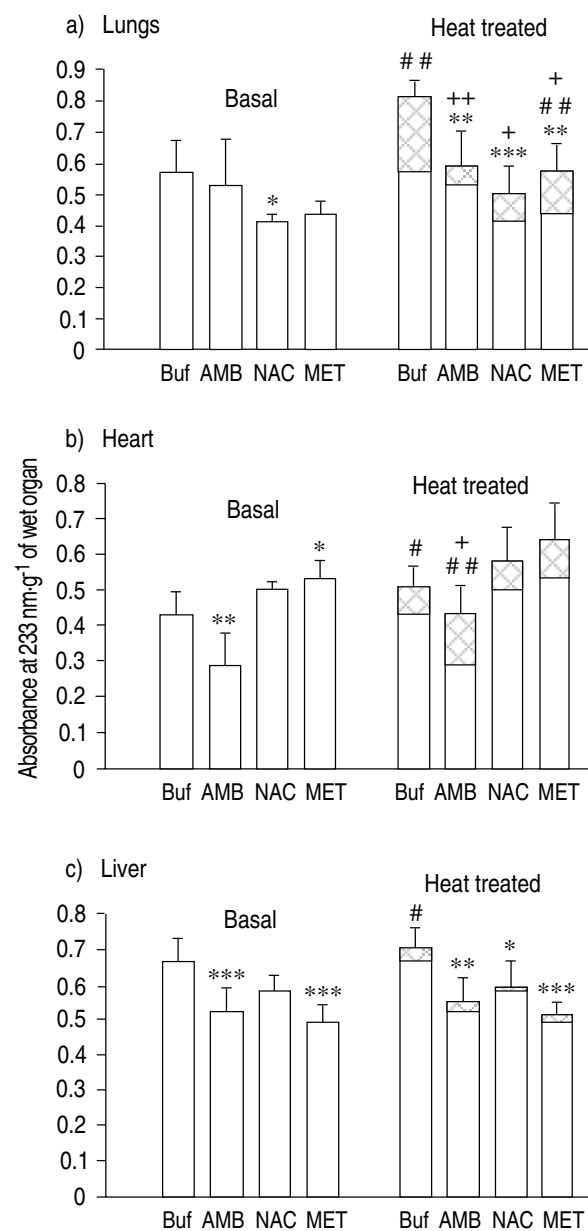

Fig. 3. – Effect of ambroxol (AMB), N-acetylcysteine (NAC) and methionine (MET) on the heat-induced increase in the content of conjugated dienes (CDs) in: a) lungs; b) heart; and c) liver of mice. Mice were injected intraperitoneally with AMB (70 mg·kg⁻¹), NAC (27.6 mg·kg⁻¹), MET (25.2 mg·kg⁻¹) or 1 ml buffer (Buf), once a day for three consecutive days. The generation of CDs, expressed as absorbance readings at 233 nm·g⁻¹ of wet organ, was induced by incubation of the appropriate organ homogenate for 60 min at 50°C. Each value represents mean±SD of five studies with organs from five mice. ⬚ : indicates increment, above basal, in CD level with heat treatment. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$ CD level *versus* equivalent buffer control. #: $p < 0.05$; ##: $p < 0.01$ significance of increment in the content of CDs; +, ++: $p < 0.05$, 0.01 increment sig. different from buffer control.
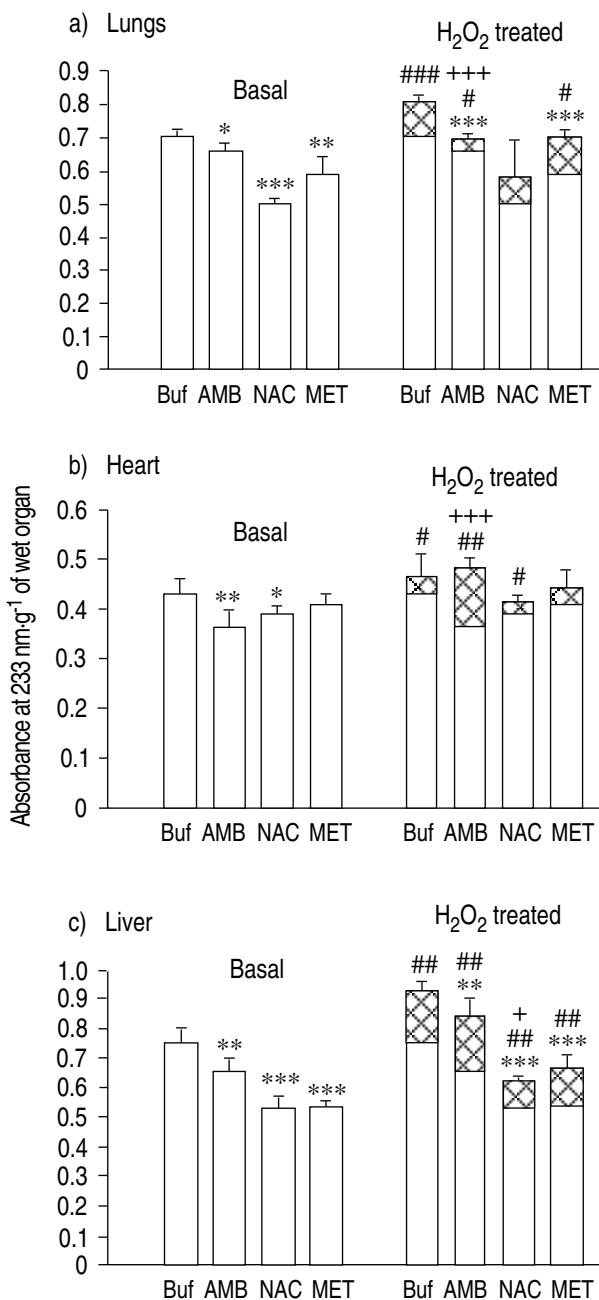
Fig. 4. – Effect of ambroxol (AMB), N-acetylcysteine (NAC) and methionine (MET) on H_2O_2-induced increase in the content of conjugated dienes (CDs) in: a) lungs; b) heart; and c) liver of mice. Mice were injected intraperitoneally with AMB (70 mg·kg⁻¹), NAC (27.6 mg·kg⁻¹), MET (25.2 mg·kg⁻¹) or 1 ml buffer (Buf), once a day for three consecutive days. The generation of CDs, expressed as absorbance readings at 233 nm·g⁻¹ of wet organ, was induced by incubation of lung, liver and heart homogenates with 10 mmol·l⁻¹ H_2O_2 for 20, 15 and 20 min respectively, at 37°C. Each value represents mean±SD of five studies with organs from five mice. ▨ : indicates increment, above basal, in CD level with H_2O_2 treatment. *: p<0.05; **: p<0.01; ***: p<0.001 CD level *versus* equivalent buffer control. #: p<0.05; ##: p<0.01; ###: p<0.001, significance of increment in the content of CDs. +, ++, +++: p<0.05, 0.01, 0.001 increment sig. different from buffer control.

Effect of drug treatment on the heat-induced generation of CD in lung, heart and liver homogenates (fig. 3)

Figure 3 shows the effect of intraperitoneal administration of ambroxol, N-acetylcysteine and methionine on the heat-induced lipid peroxidation in organ homogenates. Ambroxol inhibited the thermal generation of CD in lung homogenates 3.7 fold (p<0.01) compared to lungs from buffer-treated animals (fig. 3a). The inhibition of the heat-induced CD formation caused by ambroxol (59.9±33.1%) was similar to that with N-acetylcysteine (61.1±26.1%), but 1.4 fold higher than that observed for methionine (42.6±24.3%). Thus, the lungs from ambroxol-, N-acetylcysteine- and methionine-treated mice revealed, respectively, a 1.4 (p<0.01), 1.6 (p<0.001) and 1.4 fold (p<0.01) lower content of CD after incubation at 50°C than lungs from control buffer-injected animals.

All drugs decreased the mean heat-induced increment in CD content of liver homogenates; although not significantly (fig. 3c). None of the drugs inhibited the heat-induced generation of CD in heart homogenates (fig. 3b). Moreover, ambroxol enhanced twofold (p<0.05) the mean increment in the content of CD in heart homogenates from 0.071±0.036 to 0.140±0.032 after the incubation at 50°C. However, the CD level in heat-treated hearts from ambroxol group did not differ significantly from that found for equivalent hearts harvested from control buffer-injected mice.

Effect of drug treatment on the H_2O_2-induced generation of CD in lung, heart and liver homogenates (fig. 4)

Figure 4 summarizes the effect of ambroxol, N-acetylcysteine and methionine on lipid peroxidation in organ homogenates after incubation with H_2O_2. The H_2O_2-induced generation of CD in lungs from ambroxol-treated animals was 3.1 fold lower (p<0.001) than that observed in lung homogenates from the control group (fig. 4a). Nevertheless, the mean CD increment was statistically significant, probably due to the low variability of individual results.

Methionine and N-acetylcysteine did not significantly decrease the H_2O_2-induced pulmonary increment in the content of CD. However, lungs from methionine-treated mice revealed about a 1.2 fold (p<0.001) lower content of CD after incubation with H_2O_2 than lungs from control buffer-injected animals. This may be due to reduction of basal pulmonary CD level.

Ambroxol, N-acetylcysteine, and methionine did not reduce CD H_2O_2-induced generation in heart homogenates (fig. 4b). Moreover, heart homogenates from mice treated with ambroxol showed a 3.6 fold higher (p<0.001) increment in CD than those from control animals. The heart homogenates from each drug-treated animal group did not have a higher CD content, after incubation with H_2O_2 than that from control mice.

Only N-acetylcysteine significantly inhibited, by about twofold (p<0.05), the generation of CD in liver homogenates caused by H_2O_2 (fig. 4c). However, all 3 drugs decreased the liver CD level both basal and after incubation with H_2O_2.

Discussion

In our study, we tested ambroxol as an inhibitor of the harmful lipid peroxidation process in murine lungs, and compared its activity with the two antioxidants,

N-acetylcysteine and methionine. The procedure consisted of the following steps: 1) intraperitoneal administration of the test drug in mice; 2) isolation and preparation of lung homogenates; 3) induction of lipid peroxidation in lung homogenates by heat 50°C or 10 mmol·l^{-1} H_2O_2; and 4) lung CD content measured. Slight modifications of the incubation time also allowed for evaluation of possible protective effect of the drugs on lipids from other organs, i.e. heart and liver.

We found that ambroxol protected lung lipids from both heat- and H_2O_2-induced peroxidation, indicating protection against damage. Methionine and N-acetylcysteine decreased only the heat-induced formation of CD in lung homogenates, being as effective as ambroxol.

The results are in agreement with our previous experiments showing that ambroxol was equipotent to N-acetylcysteine in inhibition of lipopolysaccharide-induced lung and heart lipids peroxidation in mice [14]. However, in this model, ambroxol could act not only as an antioxidant but also as an inhibitor of phagocyte function and cytokine release [8, 9], since it also protected animals from the lipopolysaccharide-induced hypothermic reaction [14]. Our present study clearly indicates that ambroxol can act as an efficient lipid antioxidant. Most of the deleterious effects of H_2O_2 on tissues, including lipid peroxidation, depend on its conversion into OH· which is catalysed by iron and copper [18–20]. Recently, we found that ambroxol scavenged OH· [15], and this may explain its protective effect against H_2O_2 on lung lipids. In addition, in vitro ambroxol at concentrations of 25 and 250 µM decreased OH·-induced peroxidation of linoleic acid by 18 and 66%, respectively [15].

In rats, the lung concentration of ambroxol, measured 3 min after single intravenous treatment of a 6 mg·kg^{-1} dose, was about 140 µM [21]. Our animals received an 11.6 fold higher dose of drug for three consecutive days. Thus, the pulmonary concentration of ambroxol could reach values corresponding to those able to inhibit linoleic acid peroxidation in vitro. In humans, the plasma concentration of ambroxol determined at 2.5 h after single oral administration of 90 mg drug was about 0.6 µM [22]; however, the pulmonary concentration could be several times higher.

The exact mechanism of the inhibitory effect of ambroxol on the heat-induced lipid peroxidation in lung homogenates remains unknown. Perhaps it can inhibit thermally-induced hydrogen abstraction from polyunsaturated fatty acids, resulting in the formation of CD. The influence of ambroxol on the reactivity and availability of tissue iron and copper should also be considered. It is possible that ambroxol can chelate these transition metals by the two nitrogens in the molecule, and thus inhibit lipid peroxidation.

Exposure of lung homogenates to heat and H_2O_2 increased their CD content. The CD level in lung homogenates from ambroxol-injected mice was always lower than that from buffer-treated animals. However, only in lungs subjected to action of heat was it accompanied by abolition of CD increment. In ambroxol-treated lungs incubated with H_2O_2, the CD increment was low but still

significant. This may be due to the lower variability of individual results with H_2O_2 than those obtained with heat, the more so as ambroxol caused a similar mean decrease (3.7 versus 3.1 fold) of heat- and H_2O_2-induced CD formation, respectively, in lung homogenates. However, the cause may be that of different mechanisms leading to CD generation by heat and H_2O_2. Perhaps the lung lipid peroxidation caused by a relatively high concentration of H_2O_2 (10 mmol·l^{-1}) is less susceptible to changes of antioxidant activity in separate organs of mice than that induced by heat. Therefore, the individual H_2O_2-dependent CD increments were more reproducible and, although the mean CD increment was low, it was statistically significant.

In earlier work, we found that ambroxol almost completely inhibited the endotoxin-induced generation of CD in both heart and lungs of mice [14]. The heart concentration of ambroxol after a single intravenous injection was threefold lower than that in lungs but twofold higher than that in liver [21]. In addition, the antioxidant defence of heart is lower than that of lungs and liver [23]. Therefore, we additionally evaluated the effect of administration of ambroxol on heat- and H_2O_2-induced formation of CD in heart and liver homogenates. Ambroxol had no influence on lipid peroxidation in liver, but enhanced the increment in CD content in heart homogenates induced either by H_2O_2 or heat. On the other hand, the basal CD content in heart homogenates obtained from ambroxol-treated mice was lower than that observed in control buffer-treated animals. Moreover, the final CD content (after heating and incubation with H_2O_2) in heart homogenates from ambroxol-injected mice was similar to the basal value found in the control group. Therefore, it is difficult to conclude whether ambroxol revealed the pro-oxidant effect in heart homogenates and this question requires further studies. Perhaps, the low activity of antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, in murine heart [23], and myoglobin-derived iron may be partly responsible for these observations.

As mentioned above, ambroxol decreased the basal CD content in all organ homogenates, and both N-acetylcysteine and methionine lowered the lung and liver CD level, but proved variable on heart CD level. It cannot be excluded that this may have a further influence on CD formation in organ homogenates following heat or H_2O_2 exposure. It is possible that the lower basal tissue lipid peroxidation may allow a higher increment in CD content under the conditions of our experiments, because more polyunsaturated fatty acids are available for peroxidation. Thus, to some extent, it can mask the protective effect of antioxidant. Nevertheless, ambroxol significantly decreased the heat- and H_2O_2-induced CD generation in lung homogenates. On the other hand, it seems quite clear that in vivo administration of an antioxidant can simultaneously decrease both the basal and the provoked lipid peroxidation.

Unfortunately, in many studies concerning the protection of lung lipids from peroxidative damage, the influence of antioxidants and free radical scavengers on the basal pulmonary level of lipid peroxidation products was not

determined [24–27]. However, our results are supported by the observations of HERSHKO *et al.* [28], who found that α-tocopherol decreased the basal level of malondialdehyde (a lipid peroxidation product) in myocardial cell cultures, and also inhibited iron-induced lipid peroxidation in these cells.

Our study indicates that ambroxol can act as a sufficient lung lipid antioxidant. This may be of value, in view of the fact that ambroxol is often used in adjuvant treatment of chronic bronchitis and pulmonary emphysema, which are (among others) characterized by oxidant-antioxidant imbalance in the lower airways [29, 30].

References

1. Smith LL. The response of the lung to foreign compounds that produce free radicals. *Ann Rev Physiol* 1986; 48: 681–692.
2. Pryor WA. Oxy-radicals and related species: their formation, lifetimes and reactions. *Ann Rev Physiol* 1986; 48: 657–667.
3. Cerutti P, Kapanci Y. Effects of metabolite VIII of bromhexine (Na 872) on type II epithelium of the lung. An experimental and morphological study with reference to surfactant secretion. *Respiration* 1979; 37: 241–251.
4. Winsel K. Antioxidative und entzündungshemmende Eigenschaften von Ambroxol. *Pneumologie* 1992; 46: 461–475.
5. Rozniecki J, Nowak D. Effect of ambroxol on the chloramine T-induced decrease of serum elastase inhibitory capacity *in vitro*. *Lung & Respiration* 1987; 4: 14–15.
6. Stockley RA, Shaw J, Burnett D. Effect of ambroxol on neutrophil chemotaxis *in vitro*. *Agents Actions* 1988; 24: 292–296.
7. Rozniecki J, Nowak D. Ambroxol inhibits spontaneous migration of human neutrophils. *Clin Exp Allergol* 1990; 20 (Suppl. 1): 27.
8. Winsel K, Becher G. Effects of ambroxol on chemiluminescence of phagocytic cells and Na-arachidonate-induced bronchoconstriction in guinea-pigs. *Eur Respir J* 1992; 5 (Suppl. 15): 289.
9. Bianchi M, Mantovani A, Erroi A, Dinarello CA, Ghezzi P. Ambroxol inhibits interleukin-1 and tumor necrosis factor production in human mononuclear cells. *Agents Action* 1990; 31: 275–279.
10. Luisetti M, Peona V, Salmona M, *et al*. Ambroxol and pulmonary toxicity induced by antineoplastic drugs. *Int J Clin Pharmacol Res* 1986; 6: 129–136.
11. Donnini M, Luisetti M, Diomede L, *et al*. Ambroxol reduces paraquat toxicity in the rat. *In*: Wichert P, von Muller B, eds. Basic Research on Lung Surfactant. Progress in Respiratory Research. Karger Basel, 1990; 25: pp. 329–332.
12. Nowak D, Pietras T, Antczak A, Krol M. Inhibition of endotoxin-induced lipid peroxidation by ambroxol in mice. *Lung & Respiration* 1993; 10: 14–15.

13. Bernard GR. N-acetylcysteine in experimental and clinical acute lung injury. *Am J Med* 1991; 91 (Suppl. 3C): 54–59.
14. Nowak D, Pietras T, Antczak A, Krol M, Piasecka G. Ambroxol inhibits endotoxin-induced lipid peroxidation in mice. *Pol J Pharmacol* 1993; 45: 317–322.
15. Nowak D, Antczak A, Krol M, Bialasiewicz P, Pietras T. Antioxidant properties of ambroxol. *Free Rad Biol Med* 1994; 16: 517–522.
16. Buege JA, Aust SD. Microsonal lipid peroxidation. *In*: Fleischer S, Packer L, eds. Methods in Enzymology. New York, Academic Press, 1978; 52: pp. 303–310.
17. Nowak D, Pietras T, Antczak A, Krol M, Piasecka G. Effect of bacterial lipopolysaccharide on the content of lipid peroxidation products in lungs and other organs of mice. Anton Leeuwenhoek *Int J Gen M* 1993; 63: 77–83.
18. Mello Filho AC, Hoffmann ME, Meneghini R. Cell killing and DNA damage by hydrogen peroxide are mediated by intracellular iron. *Biochem J* 1984; 218: 273–275.
19. Imlay JA, Chin SM, Linn S. Toxic DNA damage by hydrogen peroxide through the Fenton reaction *in vivo* and *in vitro*. *Science* 1988; 240: 640–642.
20. Rowley DA, Halliwell B. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals in the presence of copper salts: a physiologically significant reaction? *Arch Biochem Biophys* 1983; 225: 279–284.
21. Greischel A, Schumacher K. Gewebespiegel und Metabolitenmuster nach *i.v.* Gabe von [^{14}C]N-A 872 Cl in verschiedenen Dosierungen als Bolus bzw. Infusion bei der Ratte. Interner Bericht Dr K. Thomae GmbH, Biberach an der Riss 1986; 1: 9 (Doc Nr.: U86–0780).
22. Nobilis M, Pastera J, Svoboda D, Kvetina J. High-performance liquid chromatographic determination of ambroxol in human plasma. *J Chromatogr* 1992; 581: 251–255.
23. Doroshow IH, Locker GY, Myers CE. Enzymatic defenses of the mouse heart against reactive oxygen metabolites. *J Clin Invest* 1980; 65: 128–135.
24. Kennedy TP, Rao NV, Noah W, *et al*. Ibuprofen prevents oxidant lung injury and *in vitro* lipid peroxidation by chelating iron. *J Clin Invest* 1990; 86: 1565–1573.
25. Al-Mehdi AB, Dodia C, Jain MK, Fisher AB. A phospholipase A$_2$ inhibitor decreases generation of thiobarbituric acid reactive substances during lung ischemia-reperfusion. *Biochem Biophys Acta* 1993; 1167: 56–62.
26. Ward PA, Till GO, Hatherill JR, Annesley TM, Kunkel RG. Systemic complement activation, lung injury and products of lipid peroxidation. *J Clin Invest* 1985; 76: 517–527.
27. Till GO, Hatherill JR, Tourtellotte WW, Lutz MJ, Ward PA. Lipid peroxidation and acute lung injury after thermal trauma to skin. Evidence of a role for hydroxyl radical. *Am J Pathol* 1985; 119: 376–384.
28. Hershko C, Link G, Pinson A. Modification of iron uptake and lipid peroxidation by hypoxia, ascorbic acid, and α-tocopherol in iron-loaded rat myocardial cell cultures. *J Lab Clin Med* 1987; 110: 355–361.
29. Riley DJ, Kerr JS. Oxidant injury of the extracellular matrix: potential role in the pathogenesis of pulmonary emphysema. *Lung* 1985; 163: 1–13.
30. Stockley RA. Alpha$_1$-antitrypsin and the pathogenesis of emphysema. *Lung* 1987; 165: 61–77.