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The observed effects after ozone exposure strongly depend on ozone concentration and exposure time. We hypothesized that depending on the O₃ exposure protocol, mainly either an oxidant damage or an inflammation will determine the O₃ toxicity. We compared two different ozone exposure protocols: an acute exposure (3 ppm 2 h) for studying the oxidant damage and an exposure (1 ppm 12 h) where an inflammatory component is also probably involved. We measured LDH activity and protein and albumin exudation as markers for cellular damage. After the acute exposure an increase in LDH activity was measured and after exposure to 1 ppm ozone for 12 h the exudation of protein and albumin was also enhanced. The histological examinations showed a neutrophilic inflammatory response only after exposure to 1 ppm ozone for 12 h. The acute exposure protocol resulted in an increased release of PGE₂, PGD₂, PGF_{2α} and 6-ketoPGF_{1α} whereas exposure to 1 ppm ozone for 12 h led to an additional release of LTB₄. No effects were measured on the release of TxB₂ and LTC₄/D₄/E₄. These changed amounts of eicosanoids will probably contribute to the ozone-induced lung function changes.

Key words: Ozone, Guinea pigs, Inflammation, Neutrophils, Eicosanoids

The effect of ozone exposure on the release of eicosanoids in guinea-pig BAL fluid in relation to cellular damage and inflammation

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Introduction

Ozone is an important constituent of photochemical smog and because of its high oxidation potential is able to produce alterations in various functional, biochemical and morphological properties in the airways of humans and experimental animals.¹ Cellular membranes are sensitive to oxidative stress because they contain important targets like polyunsaturated fatty acids, sulfhydryl groups and amino acids of proteins which can easily be oxidized. Damage to cellular membranes may result in the release of lactate dehydrogenase (LDH) and an accumulation of proteins and inflammatory cells in broncho-alveolar lavage (BAL) fluid which are generally seen as biomarkers for lung injury. Also after exposure to ozone these phenomena are observed in animal as well as in human studies.^{2–7}

Prostaglandins and leukotrienes have been shown to be importantly involved in the inflammatory response in the airways^{8–12} and also

some studies have been performed to investigate the role of these lipid mediators in the ozone-induced lung function changes.^{4,13–16} However, due to relatively large interspecies differences^{17–20} concerning receptor distribution and the release of eicosanoids the obtained results are rather contradictory. Also the exposure protocols used differ widely among the studies performed to investigate the effects of ozone exposure. The main purpose of the studies carried out in our laboratories is to unravel the complexity underlying the ozone-induced changes in guinea-pig lung function. The current study deals with the release of eicosanoids in guinea-pig BAL fluid in relation to the inflammatory response. The results presented here come from two different ozone exposure protocols; an acute exposure to 3 ppm ozone for 2 h where probably only an oxidative effect is responsible for the observed damage and an exposure to a lower ozone concentration (1 ppm) over a longer period of time (12 h) where the effects are expected to

be mainly determined by an inflammatory response. Our objective is to study and compare the release of eicosanoids in both the ozone exposure protocols i.e. oxidant and inflammation mediated toxicity.

Materials and Methods

Animals

Male Dunkin-Hartley guinea pigs, weighing 300–350 g, obtained from Harlan CPB (Zeist, The Netherlands) were kept in a light- and temperature-controlled room ($21 \pm 1^\circ\text{C}$, humidity $50 \pm 5\%$). The animals were fed a standard diet (Hope Farms, Woerden, The Netherlands) and were allowed to tap water *ad libitum*. The animals were adapted to the laboratory housing conditions for at least 1 week before starting the exposure.

Animal exposure

Guinea pigs were placed separately in rectangular stainless steel inhalation chambers²¹ with a volume of 0.21 m^3 . Two different exposure conditions were used: exposure to 3 ppm (6 mg/m^3) ozone for 2 h and exposure to 1 ppm (2 mg/m^3) ozone for 12 h (guinea pigs were killed immediately after exposure). The ozone was generated by passing O_2 (pressure 1 atm.) through a pen-ray UV-light generator type 3 SC-9 with a SCT4 power-supply (Ultra-Violet Products, San Gabriel, CA, USA) in which oxygen is partially converted to ozone. The ozone was diluted with filtered clean air as it was drawn into the exposure chamber with an air flow of $6.0 \text{ m}^3/\text{h}$. The exposure chambers were conditioned at a temperature of $21 \pm 1^\circ\text{C}$ and a relative humidity of $50 \pm 5\%$. The ozone concentration within each chamber was constantly monitored using a UV-Photometric analyzer model 9810 (Monitor Labs, San Diego, CA, USA). Calibration was performed before the exposure period using a UV-photometric calibrator (Thermo Environmental Instruments, Franklin, MA, USA). To maintain the ozone concentration at the desired value, a manual control was performed (Mass Flow Controller Type AFC-260, ASM, Bilthoven, The Netherlands).

Control animals were exposed under identical conditions to clean filtered air.

Broncho-alveolar lavage (BAL)

After exposure to ozone lungs were removed, weighed and perfused using saline to remove excessive blood. Lungs were lavaged using

40 ml prewarmed (37°C) 0.9% saline per kg body weight. Three repetitive lavages with the same aliquot were performed by steady instillation and withdrawal through a tracheal cannula. The obtained BAL fluid was centrifuged for 10 min at $400 \times g$ at 4°C and the cell-free lavage fluid supernatant was used for further analysis.

Analysis of LDH, protein and albumin

Cell-free BAL fluid was analysed for total protein, albumin²² and lactate dehydrogenase (LDH). Total protein was measured using the bicinchoninic acid (BCA) protein assay reagent according to the manufacturer's instructions (Pierce, Rockford, IL, USA). LDH activity was assayed at 37°C in 50 mM Tris-buffer, pH 7.4, with 5 mM EDTA, 0.15 mM NADH and 1.22 mM pyruvate by measuring the decrease in absorbance at 340 nm.

Analysis of cyclooxygenase and lipoxygenase products from guinea-pig BAL fluid

The release of cyclooxygenase products was quantified using radioimmunoassays (RIA) for PGE_2 , $\text{PGF}_{2\alpha}$, PGD_2 , 6-keto $\text{PGF}_{1\alpha}$ and TxB_2 (antibodies from PerSeptive Diagnostics (USA), tritiated compounds from Amersham (UK), and standards from Sigma Chemicals (Belgium)) according to the manufacturer's instructions. Lipoxygenase products (LTB_4 and $\text{LTC}_4/\text{D}_4/\text{E}_4$) were measured using an enzyme immunoassay (EIA) system (Amersham, UK), according to the manufacturer's instructions. Samples were assayed in duplicate and standard curves were run with each assay of unknown samples. The production of cyclooxygenase and lipoxygenase products was expressed as picograms per millilitre (pg/ml) BAL fluid.

Histological examination

After exposure to ozone the lungs were removed and inflated with a 2% solution of glutaraldehyde in 0.1 M phosphate buffer for fixation. After embedding in paraffin, $5 \mu\text{m}$ lung sections were stained with haematoxylin and eosin and examined by light microscopy.

Data analysis

Each experiment was performed in duplicate and results were statistically evaluated using Student's *t*-test. $P < 0.05$ was considered significant.

Results

LDH, protein and albumin measurements

Both exposure protocols (i.e. 3 ppm ozone for 2 h and 1 ppm ozone for 12 h) increased the amount of lactate dehydrogenase as measured in the guinea-pig BAL fluid (Table 1). The amount of total protein, or more specifically albumin, measured in the BAL fluid was not changed after exposure to 3 ppm ozone for 2 h but a significant increase was measured after exposure to 1 ppm ozone for 12 h.

Leukotriene and prostaglandin measurements

After exposure to 3 ppm ozone for 2 h the release of $\text{PGF}_{2\alpha}$, PGE_2 , PGD_2 and 6-keto $\text{PGF}_{1\alpha}$ was increased significantly compared with their respective controls ($\text{PGF}_{2\alpha}$: 1156 ± 122 pg/ml vs. 194 ± 42 pg/ml, PGE_2 : 224 ± 20 pg/ml vs. 20 ± 5 pg/ml, PGD_2 : 133 ± 26 pg/ml vs. 0 ± 0 pg/ml (not detectable) and 6-keto $\text{PGF}_{1\alpha}$: 832 ± 103 pg/ml vs. 428 ± 50 pg/ml) (Fig. 1). After exposure to 1 ppm ozone for 12 h comparable effects were measured ($\text{PGF}_{2\alpha}$: 831 ± 87 pg/ml vs. 181 ± 21 pg/ml; PGE_2 : 161 ± 18 pg/ml vs. 39 ± 10 pg/ml; PGD_2 : 117 ± 10 pg/ml vs. 0 ± 0 pg/ml (not detectable) and 6-keto $\text{PGF}_{1\alpha}$: 1210 ± 170 pg/ml vs. 604 ± 73 pg/ml) but after this exposure protocol also the amount of LTB_4 in the BAL fluid was increased (31 ± 2 pg/ml vs. 10 ± 3 pg/ml). No effects were measured on the release of TxA_2 (measured as TxB_2 : 4231 ± 730 pg/ml vs. 4336 ± 1262 pg/ml (3 ppm 2 h) and 4494 ± 310 pg/ml vs. 3402 ± 811 pg/ml (1 ppm 12 h) and $\text{LTC}_4/\text{D}_4/\text{E}_4$ (46 ± 7 pg/ml vs. 44 ± 5 pg/ml (3 ppm 2 h) and 45 ± 12 pg/ml vs. 35 ± 6 pg/ml (1 ppm 12 h)).

Table 1. The effect of ozone exposure (3 ppm ozone for 2 h and 1 ppm ozone for 12 h) on cellular damage markers as measured in guinea-pig BAL fluid. Values represent mean \pm SEM ($n = 5$) and *indicates $P < 0.05$

	LDH (U/l)	Total protein (mg/l)	Albumin (mg/l)
3 ppm, 2 h			
Control	72.4 ± 7.5	632 ± 118	347 ± 69
Ozone exposed	$262.2 \pm 17.9^*$	831 ± 33	379 ± 13
1 ppm, 12 h			
Control	106.4 ± 9.0	495 ± 88	221 ± 40
Ozone exposed	$334.0 \pm 32.7^*$	$1677 \pm 204^*$	$566 \pm 70^*$

Histological examination

In Fig. 2 the histological changes in guinea-pig lung tissue after ozone exposure are shown. In the control situation (Fig. 2A) a bronchiole, covered with cuboidal epithelium, is shown in cross-section together with expanded alveoli separated from each other by thin septa. Exposure to 3 ppm ozone for 2 h (Fig. 2B) results in a desquamation of the bronchiolar epithelium and thus a naked basement membrane. The bronchiolar lumen is filled with these desquamated epithelial cells. The alveolar lumina are empty and the alveolar septa are thin. A bronchiolitis consisting of polymorphonuclear cells (PMNs) and mononuclear cells is accompanied with a centriacinar inflammation when the animals are exposed to 1 ppm ozone for 12 h. The bronchiolar epithelial layer is intact (Fig. 2C).

Discussion

It is shown in this and other studies that exposure to ozone is attended by an inflammatory response.³⁻⁷ In this study we compared two different ozone exposure protocols (acute exposure to 3 ppm ozone for 2 h and an exposure to a lower concentration for a longer period of time (1 ppm ozone for 12 h) with respect to the inflammatory response coupled to the release of eicosanoids in the guinea-pig BAL fluid.

It is shown that the increase in the lactate dehydrogenase (LDH) activity can be seen as an early marker for ozone toxicity. LDH activity is the only biochemical marker we tested that already is affected after the acute exposure to ozone (3 ppm, 2 h). Measuring LDH activity represents an early indication for cellular damage; a slight change in membrane fluidity already causes a leakage of LDH from the alveolar cells in the lower airways. This rapid change in LDH release was also observed in an *in vitro* system using different types of cultured respiratory epithelial cells.²³ Exposure to relatively low ozone concentrations (0.5 ppm ozone for 3 h) already caused membrane injury of the epithelial cell types leading to increased lactate dehydrogenase release. Also in human studies early changes in LDH-release were measured in BAL fluid^{24,25} after ozone exposure.

The other two markers for cellular damage, albumin and protein, indicate an increased permeability leading to exudation of the constituents from serum into the airways. This exudation was measurable only after exposure to 1 ppm ozone for 12 h. The increase in serum

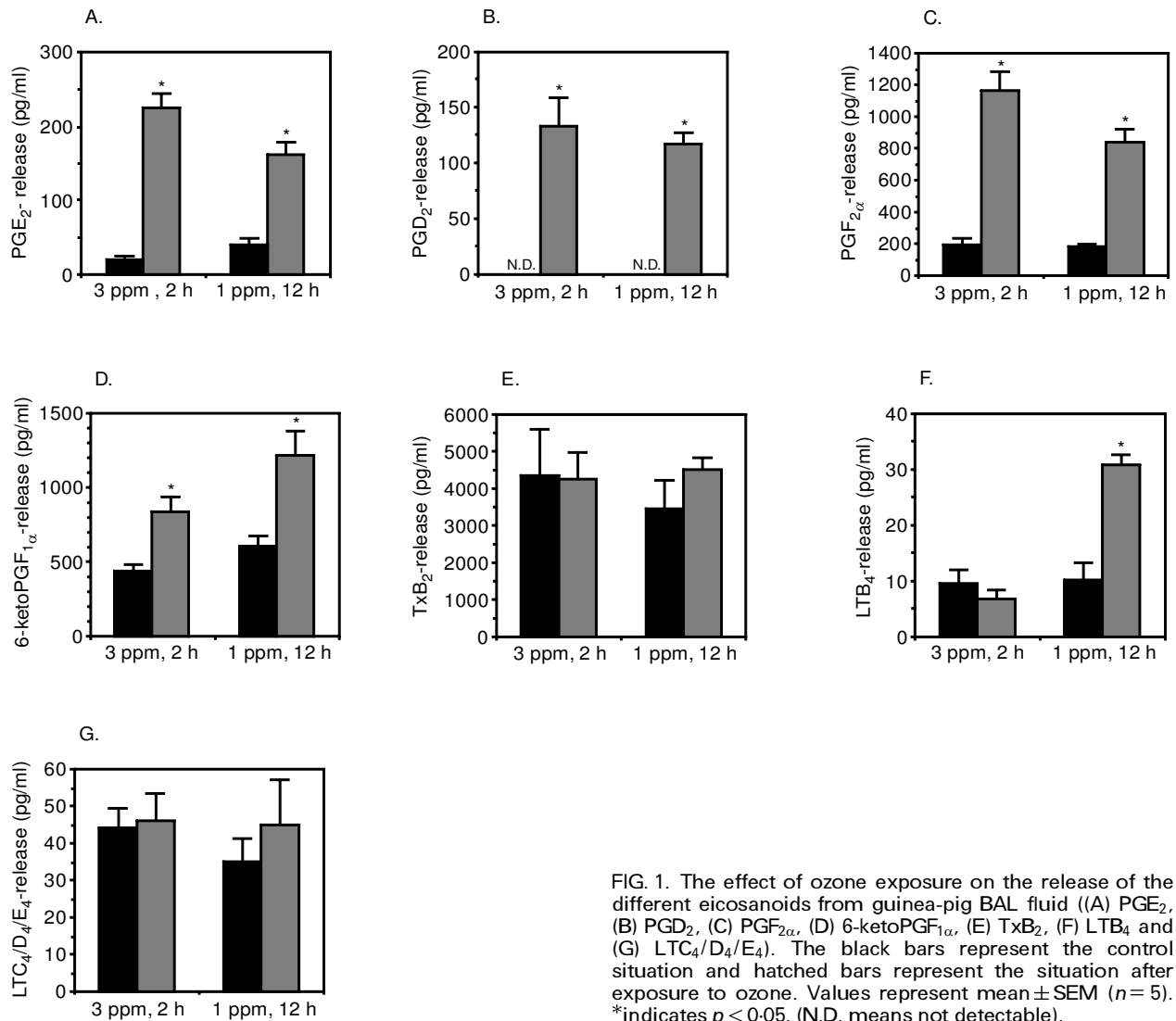


FIG. 1. The effect of ozone exposure on the release of the different eicosanoids from guinea-pig BAL fluid ((A) PGE₂, (B) PGD₂, (C) PGF_{2α}, (D) 6-ketoPGF_{1α}, (E) TxB₂, (F) LTB₄ and (G) LTC₄/D₄/E₄). The black bars represent the control situation and hatched bars represent the situation after exposure to ozone. Values represent mean ± SEM (n = 5). *indicates p < 0.05. (N.D. means not detectable).

constituents in BAL fluid may be caused by the ozone-induced oxidation of unsaturated fatty acids in lipids and susceptible amino acids in proteins, which results in an alteration of the biological membrane properties. In addition to the increased membrane permeability an influx of inflammatory cells from the blood stream into the alveolar spaces was shown in our histological studies. After exposure to 3 ppm ozone for 2 h only a desquamation of the bronchiolar epithelial layer was observed whereas after exposure to 1 ppm ozone for 12 h an infiltration of neutrophilic granulocytes could be seen. However, the bronchiolar epithelium remained intact.

Cell differentiation performed in guinea-pig broncho-alveolar lavage (BAL) fluid after exposure to these ozone exposure protocols shows comparable results (data not shown). Exposure to 1 ppm ozone for 12 h resulted in an increased amount of neutrophilic granulocytes

(represented as percentage of the total amount of cells) compared with the control situation as well as compared with the situation where the animals were exposed to 3 ppm ozone for 2 h. These current results are supported by a number of both human^{13,25} as well as animal studies. A variety of animal species has been studied in order to examine the ozone-induced injury at tissue level. Ozone-induced tissue neutrophilia was first demonstrated by Castleman *et al.*²⁶ in the bronchiolar wall of Rhesus monkeys after a 4 h exposure to 0.8 ppm ozone. Also in mongrel dogs a neutrophilic inflammation was observed in the tracheal and bronchial mucosa²⁷ already 1 h after exposure to 2.1 ppm ozone for 2 h which was accompanied by an ozone-induced hyperresponsiveness. In a study where guinea pigs were exposed to 3 ppm ozone during 2 h (identical to our acute exposure protocol) the time course of histological changes was examined.²⁸ In agreement with our findings no

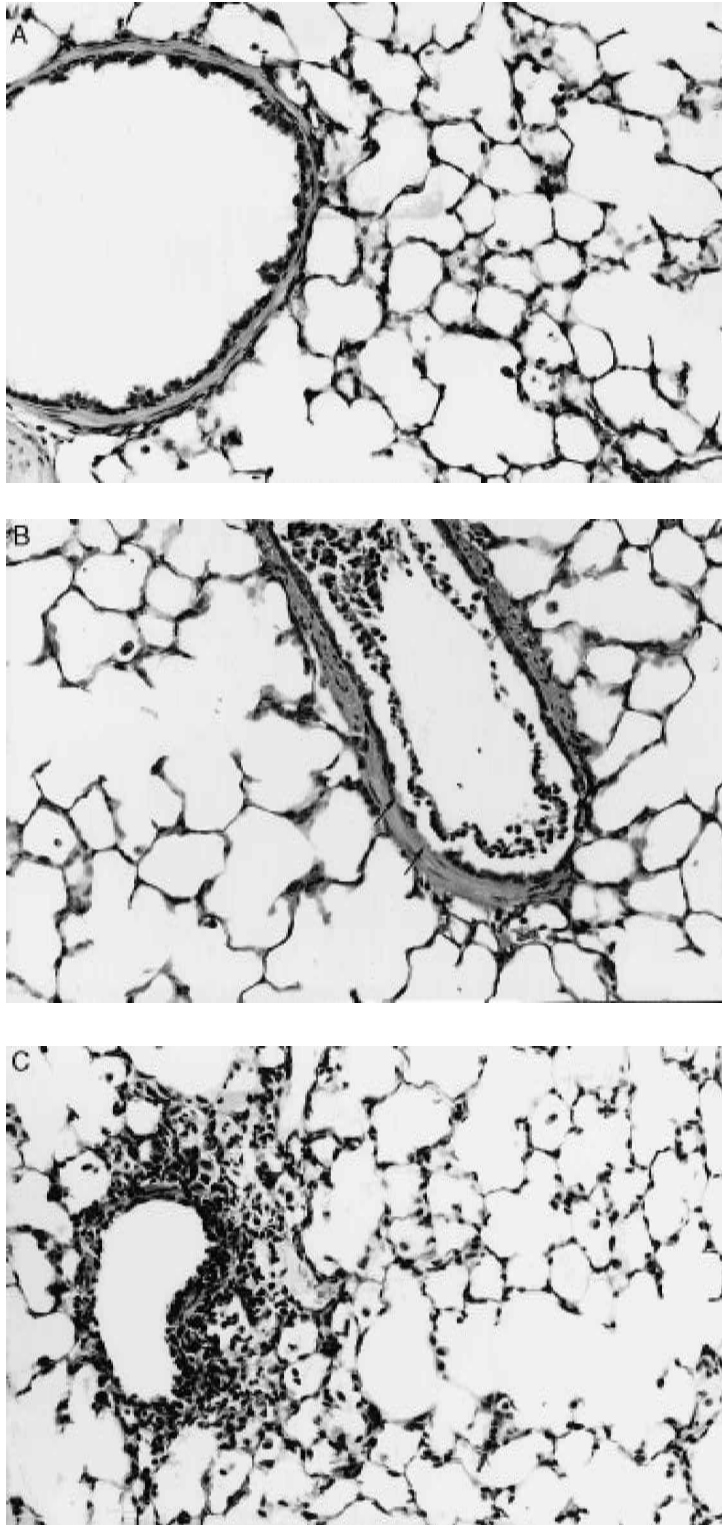


FIG. 2. Histological and morphological effects of ozone exposure on guinea-pig lung tissue. (A) Control guinea-pig lung with a bronchiole and expanded alveoli; (B) guinea-pig lung after exposure to 3 ppm ozone for 2 h: desquamation of bronchiolar epithelial cells resulted in a naked basement membrane. The lumen is filled with these epithelial cells and (C) guinea-pig lung after exposure to 1 ppm ozone for 12 h: a bronchiolitis of polymorphonuclear and mononuclear cells is shown with a centriacinar inflammation (HE, 180 \times).

inflammatory effect was observed immediately after exposure but at 6 h after exposure an increase in neutrophilic granulocytes was measurable peaking at 2 days post-exposure. Also

after an exposure to 2 ppm ozone for 4 h a rapid accumulation of polymorphonuclear leukocytes (PMNs) in guinea-pig lung interstitial and airway spaces was observed.⁵ The inflam-

matory response declined to control values within 24 h in lung interstitium whereas the increased amount of inflammatory cells measured in BAL fluid remained elevated for 3 days. Very recently a study of Sun and Fan Chung²⁹ also showed an increased number of neutrophils after single as well as after repeated exposure (exposure on 4 successive days) to 3 ppm ozone for 3 h.

The neutrophilic granulocyte is a potential source of a wide variety of mediators, including potent lipid mediators like prostaglandins, thromboxanes, leukotriene B₄ and PAF which might contribute to altered airway responses and/or exacerbation of the inflammatory response.¹¹ In the current study it was shown that after exposure to 3 ppm ozone for 2 h a significant increase in the release of PGF_{2α}, PGE₂, PGD₂ and 6-ketoPGF_{1α} (the stable endproduct of PGI₂) in guinea-pig BAL fluid was observed. After exposure to a lower concentration over a longer period of time (1 ppm ozone for 12 h) an additional increased release of LTB₄ was measured. No effects were observed concerning the release of TxB₂ (stable end product of TxA₂) and LTC₄/D₄/E₄.

Although no neutrophilic inflammatory response was observed after exposure to 3 ppm ozone for 2 h, an increased release of some of the lipid mediators was perceived. This observation suggests that the increase in prostaglandins is not coming from the influx of inflammatory cells, but from cells that are present in the airways under normal conditions, possibly the alveolar macrophages or airway epithelial cells.³⁰

It has been shown that LTB₄ is a predominant neutrophil chemoattractant^{31,32} which is present in alveolar macrophages and alveolar epithelial cells^{9,30} and is thought to be responsible for initiating the inflammatory response after ozone exposure.³³ The 5-lipoxygenase pathway in neutrophils selectively generates LTB₄ upon stimulation with a variety of stimuli.^{30,34} This could explain the observation that after exposure to 1 ppm ozone for 12 h, when an inflammatory response is present, a three-fold increase in the release of LTB₄ was observed whereas after exposure to 3 ppm ozone for 2 h no effects were measurable.

In our study it was shown that ozone exposure did not affect the release of LTC₄/D₄/E₄. Comparable results were found in a series of experiments where humans were exposed to 0.10 ppm ozone or 0.08 ppm ozone for 6.6 h with moderate exercise (40 l/min) and where BAL was performed 18 h after exposure.²⁵ No effect was observed in the release

of LTB₄ although a marked inflammatory response was present. The authors suggest that the time course between ozone exposure and BAL measurements (18 h) may be responsible for this observation, since neutrophils are already attracted to the lung as early as 3 h following ozone exposure.¹³ It is possible that LTB₄ is present in BAL fluid shortly after ozone exposure. Although no clear indication can be found in the literature it may be expected that comparable with the prostaglandin release¹⁷⁻²⁰ in the case of leukotriene release a species difference may also account for these observed differences between guinea pig and human studies.

LTB₄ itself is not able to contract or relax the airways but it seems able to induce airway hyperresponsiveness by the release of TxB₂.³¹ After LTB₄ inhalation an influx of neutrophils into the airways was observed but also a striking increase in TxB₂ in lavage fluid in dogs. This increased airway responsiveness was prevented by pretreatment with the thromboxane synthase inhibitor OKY-046 whereas the inhibitor did not change the amount of inflammatory cells after LTB₄ inhalation.³¹

Surprisingly, our study did not show any increase in the release of TxB₂ although an increased release of LTB₄ was present after exposure to 1 ppm ozone for 12 h. The amounts of TxB₂ measured in our experiments are, compared with the other eicosanoids, rather high. This might suggest that the neutrophils are not the major source of TxB₂ in our experimental set-up and that basal release of TxB₂ from other cell types in the airways³⁵ exceeds the release from neutrophils. On the other hand, the release of TxB₂ from thrombocytes may also account for the observed effects since these cells produce very large amounts of TxB₂^{36,37} and this might overwhelm the ozone-induced changes in the TxB₂ release.

In summary, we have shown in this study that the inflammatory response after exposure to ozone strongly depends on ozone concentration and exposure time. LDH seems to be the most sensitive marker for ozone-induced tissue damage whereas the exudation of albumin and protein is only measurable after exposure over a longer period of time. This exudation is accompanied by a neutrophilic inflammatory response and a subsequent increase in the LTB₄ release in guinea-pig BAL fluid. The other mediators remained unchanged (TxB₂ and LTC₄/D₄/E₄) or were already increased after exposure to 3 ppm ozone for 2 h in the absence of an inflammatory response (PGE₂, PGD₂, PGF_{2α} and 6-keto-PGF_{1α}). The precise role of these eicosanoids in

the ozone-induced changes in airway reactivity in our experimental set-up and exposure protocol requires further investigation.

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