

# Respiratory pathophysiologic responses

## 13-Hydroxy-linoleic acid induces airway hyperresponsiveness to histamine and methacholine in guinea pigs in vivo

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*The influence of 13-hydroxy-linoleic acid (13-HODE) on the pulmonary resistance and dynamic compliance of guinea pigs in vivo was determined. Intravenously administered histamine and methacholine caused dose-dependent increases in pulmonary resistance and dose-dependent decreases in dynamic compliance in anesthetized, spontaneously breathing guinea pigs. Inhalation of an aerosol containing 13-HODE (10  $\mu\text{mol/L}$ ) enhanced the increases in pulmonary resistance observed after administration of histamine or methacholine when compared with the respective control animals. The effect of 13-HODE on the increase in pulmonary resistance after administration of histamine was dose-dependent. An enhancement in the pulmonary resistance was already measured after treatment of guinea pigs with aerosols of solutions containing 0.1  $\mu\text{mol/L}$  13-HODE when compared with that of control animals. In contrast, the changes in dynamic compliance were not affected by 13-HODE. These results indicate that 13-HODE may play an important role in the induction of airway hyperresponsiveness in vivo when it is produced or released in significant amounts in the airways. (J ALLERGY CLIN IMMUNOL 1995;96:36-43.)*

**Key words:** Airway hyperresponsiveness, 13-HODE, linoleic acid, histamine, methacholine

Airway hyperresponsiveness and inflammation are characteristic features of asthma. The inflammatory cells and their mediators may play an important role in the induction and maintenance of airway hyperresponsiveness. Arachidonic acid-derived metabolites are an important class of substances, which are produced by inflammatory and resident cells in the lung and which can affect airway responsiveness. Prostaglandin E<sub>2</sub>, for example, relaxes airway smooth muscle and modulates the intensity of the contractions induced by several

### Abbreviations used

|           |                              |
|-----------|------------------------------|
| 9-HODE:   | 9-Hydroxy-linoleic acid      |
| 13-HODE:  | 13-Hydroxy-linoleic acid     |
| 13-HPODE: | 13-Hydroperoxy-linoleic acid |
| LT:       | Leukotriene                  |

contractile agents.<sup>1-4</sup> Leukotriene (LT)C<sub>4</sub> and LTD<sub>4</sub> cause long-lasting contractions of airway smooth muscles and increase the responsiveness of the airways to other bronchoconstrictors.<sup>5,6</sup> Arachidonic acid metabolites have also been shown to influence tracheal relaxation induced by  $\beta$ -adrenergic agents.<sup>7</sup>

Little attention has been paid to the metabolism of linoleic acid and the effects of its metabolites in the airways. Linoleic acid can be converted into 9-hydroxy-linoleic acid (9-HODE) or 13-hydroxy-linoleic acid (13-HODE) by the same enzyme systems that metabolize arachidonic acid into biologically active mediators. 9-HODE and 13-HODE can be produced by several cell types such as bronchoalveolar cells,<sup>8,9</sup> leukocytes,<sup>10-12</sup> airway

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epithelial cells,<sup>13, 14</sup> and vascular endothelial cells.<sup>15</sup> Both 9-HODE and 13-HODE interfere with the activity of reactive oxygen species released by pulmonary macrophages<sup>16, 17</sup> and possess chemotactic activity for polymorphonuclear leukocytes,<sup>18</sup> whereas 13-HODE modulates arachidonic acid metabolism in platelets,<sup>19</sup> leukocytes,<sup>20</sup> and endothelial cells<sup>21, 22</sup>; affects intracellular calcium concentrations in polymorphonuclear leukocytes<sup>23</sup> and vascular smooth muscle cells<sup>24</sup>; attenuates LTB<sub>4</sub>-induced edema formation<sup>25</sup>; and maintains the endothelial lining in a nonadhesive state.<sup>15</sup>

In light of the fact that 13-HODE can be produced by different cell types in the lungs, we wondered whether 13-HODE is also of importance in the regulation of airway function and therefore studied the effects of 13-HODE on pulmonary resistance and dynamic compliance of the guinea pig *in vivo*.

## METHODS

### Animals

Male, specified pathogen-free Dunkin Hartley guinea-pigs (450 to 700 gm) were obtained from Harlan/ Porcellus (Heatfield, U.K.). All animals received water and commercial chow *ad libitum*. They were killed by an intraperitoneal injection of pentobarbital sodium (Ceva, Paris, France).

### Preparation of 13-HODE

13-HODE was obtained from Oxford Biomedical Research Inc. (Oxford, Mich.) or prepared as described by Garssen et al.<sup>26</sup> Briefly, linoleic acid (Sigma Chemical Company, St. Louis, Mo.) was incubated with lipoxygenase-1 enzyme (type IV, Sigma) at 4° C and a pH of 9. The formation of the linoleic acid intermediate 13-hydroperoxy-linoleic acid (13-HPODE) was followed by measurement of samples of the incubation mixture at appropriate intervals in a spectrophotometer (235 nm). After the linoleic acid had been metabolized, the incubation was stopped by adding hydrogen chloride to obtain a pH of 4. 13-HPODE was separated from nonmetabolized linoleic acid by means of thin-layer chromatography and was subsequently reduced with sodium borohydride. After reduction, the purity of 13-HODE was measured by reverse-phase high-performance liquid chromatography and was found to be 95% or greater. The formed 13-HODE was then stored in methanol at -80° C.

### Measurement of airway responsiveness and nebulization procedure

Guinea pigs were anesthetized with urethane and allowed to breathe spontaneously. The pulmonary resistance and dynamic lung compliance were determined, breath by breath, with a computerized respiratory ana-

lyzer.<sup>27</sup> Changes in pulmonary resistance are caused by physical obstruction in the airways, and lung compliance is indicative of the elasticity of the lungs. The trachea was cannulated and connected to a pneumotachograph by a Fleisch flow head (Depex B. V., De Bilt, The Netherlands). Two dose-response curves with histamine (0.4 to 2.0 µg/100 gm body weight, administered intravenously) or methacholine (0.2 to 1.2 µg/100 gm body weight, administered intravenously) were recorded in each animal, one before and one after the administration of 13-HODE or vehicle. 13-HODE in methanol was diluted in saline solution to yield concentrations of 0.1 to 10 µmol/L, whereas control solutions contained 0.1% methanol. These solutions were applied to the airways of the animals as aerosols generated by a Kalwar model 5 ultrasonic nebulizer (Stöpler, Utrecht, The Netherlands), which produced droplets with a mass median aerodynamic diameter of about 2 to 3 µm. The nebulization rate was 0.5 ml solvent per minute, which was mixed with 1.6 m<sup>3</sup> filtered room air per hour. The aerosols were allowed to flow into a 1 L box, which was coupled, by a T valve in front of the flow head, to the cannula in the trachea of each animal. The animals were allowed to inhale the aerosols for 5 respiratory cycles. This procedure was repeated 5 times every 5 minutes. Directly after the administration of 13-HODE or control aerosol, the second histamine or methacholine dose-response curve was performed.

### Morphologic examination

Guinea pigs were anesthetized and treated with control or 13-HODE (10 µmol/L) aerosols. After the treatments, the lungs were removed from the guinea pigs (after lethal anesthesia with pentobarbital) and filled intratracheally with formalin fixing solution (0.8%). The unfolded lungs were placed in the fixative for at least 24 hours, dehydrated, and embedded in paraplast (Monoject, Kildare, Ireland). Sections of 4 µm in thickness were stained with hematoxylin and eosin and evaluated for the presence of inflammatory cells. Three animals from each treatment group were studied.

### Statistical analysis

Data are reported as the mean of three or more independent observations ± standard error of the mean. Significance was assessed with Student's *t* test. Probabilities less than 0.05 were accepted as indicating significant differences.

## RESULTS

Both histamine and methacholine caused dose-dependent increases in pulmonary resistance and dose-dependent decreases in dynamic compliance. The changes in dynamic compliance were not influenced by inhalation of 13-HODE aerosols by the guinea pigs (Table I). Also, the basal pulmonary resistance values after administration of control or 13-HODE aerosols (0.25 ± 0.05 and 0.23 ±

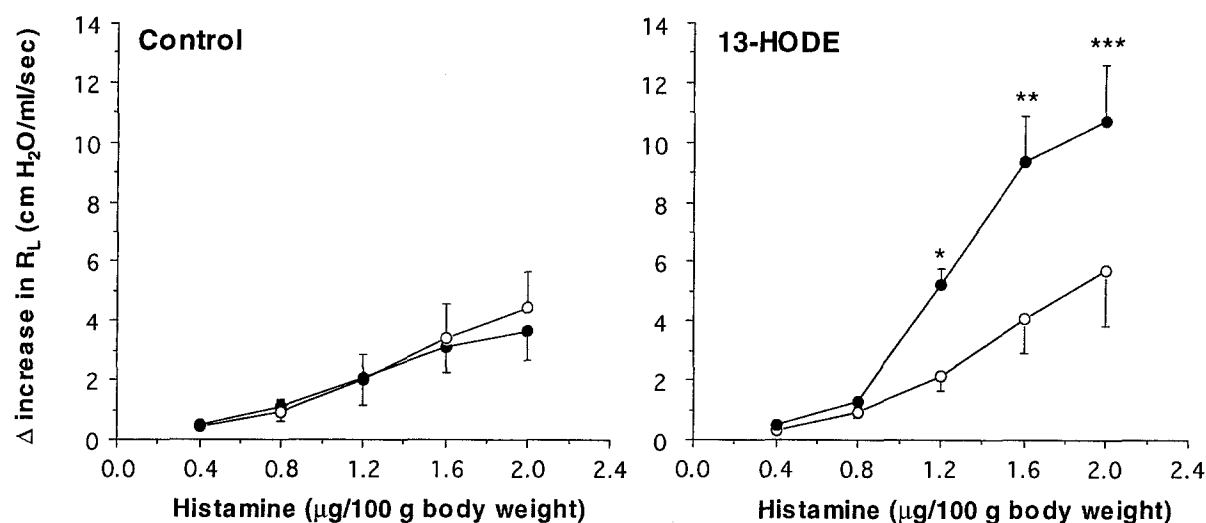


FIG. 1. Histamine-induced changes in pulmonary resistance in 13-HODE-treated and control animals before (open circles) and after (filled circles) aerosol administration ( $n = 3$ ). Values represent means  $\pm$  SEM. \* $p < 0.05$ , \*\* $p < 0.005$ , and \*\*\* $p < 0.001$  when compared with values obtained before 13-HODE administration (Student's paired  $t$  test).  $R_L$ , Pulmonary resistance.

TABLE I. Histamine- and methacholine-induced changes in dynamic compliance in 13-HODE-treated and control animals before and after aerosol administration

|                                              | Control aerosol |                 | 13-HODE aerosol |                 |
|----------------------------------------------|-----------------|-----------------|-----------------|-----------------|
|                                              | Before          | After           | Before          | After           |
| <b>Histamine</b>                             |                 |                 |                 |                 |
| ( $\mu\text{g}/100 \text{ gm body weight}$ ) |                 |                 |                 |                 |
| 0.0 $\mu\text{g}$                            | $0.54 \pm 0.03$ | $0.50 \pm 0.03$ | $0.52 \pm 0.04$ | $0.58 \pm 0.03$ |
| 0.4 $\mu\text{g}$                            | $0.30 \pm 0.03$ | $0.25 \pm 0.02$ | $0.32 \pm 0.06$ | $0.29 \pm 0.07$ |
| 0.8 $\mu\text{g}$                            | $0.11 \pm 0.02$ | $0.11 \pm 0.01$ | $0.15 \pm 0.05$ | $0.13 \pm 0.04$ |
| 1.2 $\mu\text{g}$                            | $0.04 \pm 0.02$ | $0.05 \pm 0.02$ | $0.09 \pm 0.05$ | $0.05 \pm 0.04$ |
| 1.6 $\mu\text{g}$                            | $0.01 \pm 0.01$ | $0.01 \pm 0.01$ | $0.03 \pm 0.02$ | $0.01 \pm 0.01$ |
| 2.0 $\mu\text{g}$                            | $0.00 \pm 0.00$ | $0.00 \pm 0.00$ | $0.00 \pm 0.00$ | $0.00 \pm 0.00$ |
| <b>Methacholine</b>                          |                 |                 |                 |                 |
| ( $\mu\text{g}/100 \text{ gm body weight}$ ) |                 |                 |                 |                 |
| 0.0 $\mu\text{g}$                            | $0.78 \pm 0.17$ | $0.72 \pm 0.17$ | $0.74 \pm 0.09$ | $0.65 \pm 0.10$ |
| 0.2 $\mu\text{g}$                            | $0.53 \pm 0.08$ | $0.51 \pm 0.08$ | $0.55 \pm 0.10$ | $0.42 \pm 0.07$ |
| 0.4 $\mu\text{g}$                            | $0.42 \pm 0.07$ | $0.37 \pm 0.07$ | $0.45 \pm 0.09$ | $0.33 \pm 0.09$ |
| 0.6 $\mu\text{g}$                            | $0.20 \pm 0.06$ | $0.19 \pm 0.06$ | $0.30 \pm 0.08$ | $0.21 \pm 0.07$ |
| 0.8 $\mu\text{g}$                            | $0.14 \pm 0.04$ | $0.13 \pm 0.05$ | $0.18 \pm 0.08$ | $0.11 \pm 0.07$ |
| 1.0 $\mu\text{g}$                            | $0.05 \pm 0.02$ | $0.06 \pm 0.03$ | $0.08 \pm 0.05$ | $0.05 \pm 0.04$ |
| 1.2 $\mu\text{g}$                            | $0.01 \pm 0.01$ | $0.01 \pm 0.01$ | $0.01 \pm 0.00$ | $0.00 \pm 0.00$ |

Values represent mean dynamic compliance in milliliters per centimeter  $\text{H}_2\text{O} \pm \text{SEM}$  ( $n = 4$  to 6).

0.03 cm  $\text{H}_2\text{O}/\text{ml}/\text{sec}$ , respectively) were not different from each other and were similar to the values obtained before the aerosol treatments ( $0.18 \pm 0.04$  and  $0.21 \pm 0.03$  cm  $\text{H}_2\text{O}/\text{ml}/\text{sec}$ , respectively). However, the changes in pulmonary resistance caused by histamine or methacholine were affected by treating the animals with aerosols containing 10  $\mu\text{mol}/\text{L}$  13-HODE (Figs. 1, B and 2, B). The

increase in pulmonary resistance of 13-HODE-treated animals was significantly enhanced after administration of 1.2, 1.6, or 2.0  $\mu\text{g}$  histamine/100 gm body weight (Fig. 1, B) and 1.0 or 1.2  $\mu\text{g}$  methacholine/100 gm body weight (Fig. 2, B) when compared with guinea pigs treated with aerosols of saline solution containing 0.1% methanol.

It is difficult to determine the exact amount of

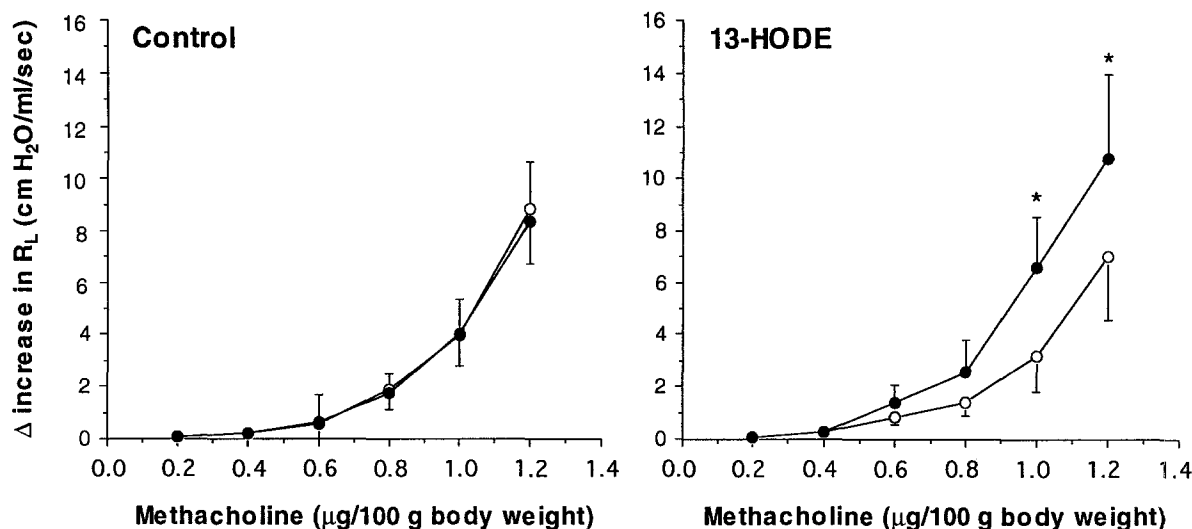


**FIG. 2.** Methacholine-induced changes in pulmonary resistance in 13-HODE-treated and control animals before (*open circles*) and after (*filled circles*) aerosol administration ( $n = 6$ ). Values represent means  $\pm$  SEM. \* $p < 0.05$  when compared with values obtained before 13-HODE administration (Student's paired  $t$  test).  $R_L$ , Pulmonary resistance.

13-HODE inhaled by the animals, but it is possible to make an estimation. The aerosols (10  $\mu\text{mol/L}$  13-HODE) contained 18.5 nmol 13-HODE solution per 100 ml of air, and because the animals inhaled 75 ml of the aerosols, about 14 nmol 13-HODE was delivered to the lungs (30 respiratory cycles; volume of respiration of the guinea pigs, approximately 3.5 ml; dead volume of the connection between the trachea and the aerosol box, 1 ml). Approximately 8% to 10% of this amount of 13-HODE will be captured in the airways. In contrast to the 13-HODE-treated animals, no differences between the first and second curve in response to histamine or methacholine were measured when the animals had inhaled the control aerosol (Figs. 1, *A* and 2, *A*). There were also no significant differences between the dose-response curves of the two groups of animals before the treatments with control solution or 13-HODE (Figs. 1 and 2).

The effect of 13-HODE on the increase in pulmonary resistance after administration of histamine was dependent on the amount of 13-HODE used (Fig. 3). An enhancement in the pulmonary resistance was already measured after treatment of guinea pigs with aerosols of solutions containing 0.1  $\mu\text{mol/L}$  13-HODE when compared with control animals (Fig. 3).

The treatment with 13-HODE did not induce inflammatory reactions in the upper and lower parts of the pulmonary system in the guinea pigs (Fig. 4). In some cases some infiltration of poly-

morphonuclear leukocytes occurred. However, this infiltration was very limited and did not differ from the limited infiltration of inflammatory cells in control animals (Fig. 4).

## DISCUSSION

This study demonstrated that 13-HODE dose-dependently induced an increased response of the airways to the contractile agents histamine and methacholine. Aerosols of a 0.1  $\mu\text{mol/L}$  13-HODE solution (corresponding with 0.14 nmol 13-HODE delivered to the lungs) were already able to increase the reactivity to histamine. The augmented reaction, as measured by the potentiated increase in pulmonary resistance, points to hyperresponsiveness of the central respiratory airways. Dynamic compliance and pulmonary resistance were measured directly after the end of the administration of aerosols to the animals. No further time-course experiments were carried out to determine how long the hyperresponsiveness was present in this *in vivo* model. However, in another series of experiments, we treated guinea pigs *in vivo* with 13-HODE or control aerosols and measured tracheal reactivity *in vitro* after different periods. We found that the increased response to histamine was still detectable *in vitro* 24 hours after the 13-HODE administration *in vivo* (unpublished results).

Airway hyperresponsiveness to bronchoconstrictor agents and airway inflammation are characteristic features of asthma, but the exact mechanisms

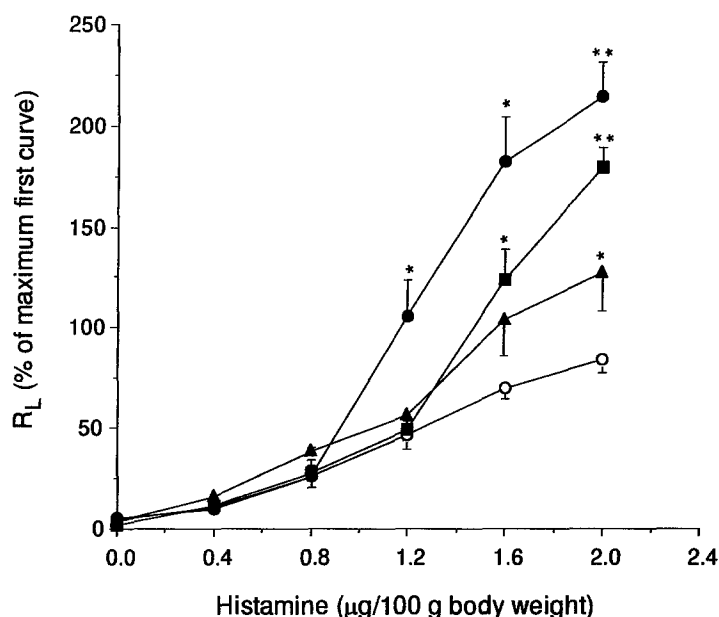


**FIG. 3.** Histamine-induced changes in pulmonary resistance after administration of aerosols containing control (open circles), 0.1 (filled triangles), 1.0 (filled squares), or 10 (filled circles)  $\mu\text{mol/L}$  13-HODE solutions. Results are expressed as percentage of maximal control values obtained before aerosol administrations  $\pm$  SEM ( $n = 3$ ). \* $p < 0.05$ , \*\* $p < 0.005$  when compared with control values (Student's unpaired  $t$  test).  $R_L$ , Pulmonary resistance.

by which bronchial hyperresponsiveness is induced are not known. Many factors may be involved such as diminished release of epithelium-derived relaxing factors and effects of mediators released, for example, by pulmonary macrophages, eosinophils, neutrophils, lymphocytes, mast cells, and sensory neurons. At present one can only speculate about the mechanisms by which 13-HODE is able to induce the observed airway hyperresponsiveness in the guinea pig. The effects induced by 13-HODE are probably not caused by an induction of edema formation or an increased mucus production in the lungs because the baseline values of the pulmonary resistance were not influenced by the 13-HODE treatment.

A possible explanation is interference of 13-HODE with the production or release of epithelium-derived relaxing factors. It has been shown that prostaglandin  $E_2$ <sup>1</sup> and nitric oxide<sup>28</sup> are produced by the airway epithelium of the guinea pig and diminish the contractile response of the smooth muscle to histamine and methacholine. It is possible that 13-HODE inhibits the release of these relaxant factors from the airway epithelial cells. In other cell types, it has been shown that 13-HODE inhibits the production of metabolites of arachidonic acid.<sup>19, 20</sup>

Fatty acid-derived metabolites, including 13-

HODE, are lipophilic mediators, which can alter the surface active properties of the airways or be incorporated into plasma membranes. This incorporation could alter membrane fluidity, thereby influencing the response of the airway tissues to contractile and relaxing agonists. In general, membrane lipid composition influences the recognition sites for agonists, the three-dimensional structure of membrane receptors, and hence agonist affinities and the production of second messengers. The linoleic acid metabolite HPODE is able to change the plasma membrane potential of rat alveolar macrophages.<sup>17</sup> Also, respiratory histamine and cholinergic receptor functions are altered in isolated tracheal preparations of guinea pigs receiving diets varying in linoleic acid content.<sup>29</sup> It is possible that the observed changes in airway reactivity after 13-HODE treatment are due to changes in plasma membrane composition, leading to an increased response to histamine and methacholine.

Another possible mechanism by which 13-HODE could influence airway responsiveness is through the induction of release of mediators from mast cells, pulmonary macrophages, or sensory neurons, which in turn could change the reactivity to the contractile agonists. 13-HODE, for example, inhibits the activity of reactive oxygen species produced by pulmonary macrophages.<sup>16, 17</sup> At

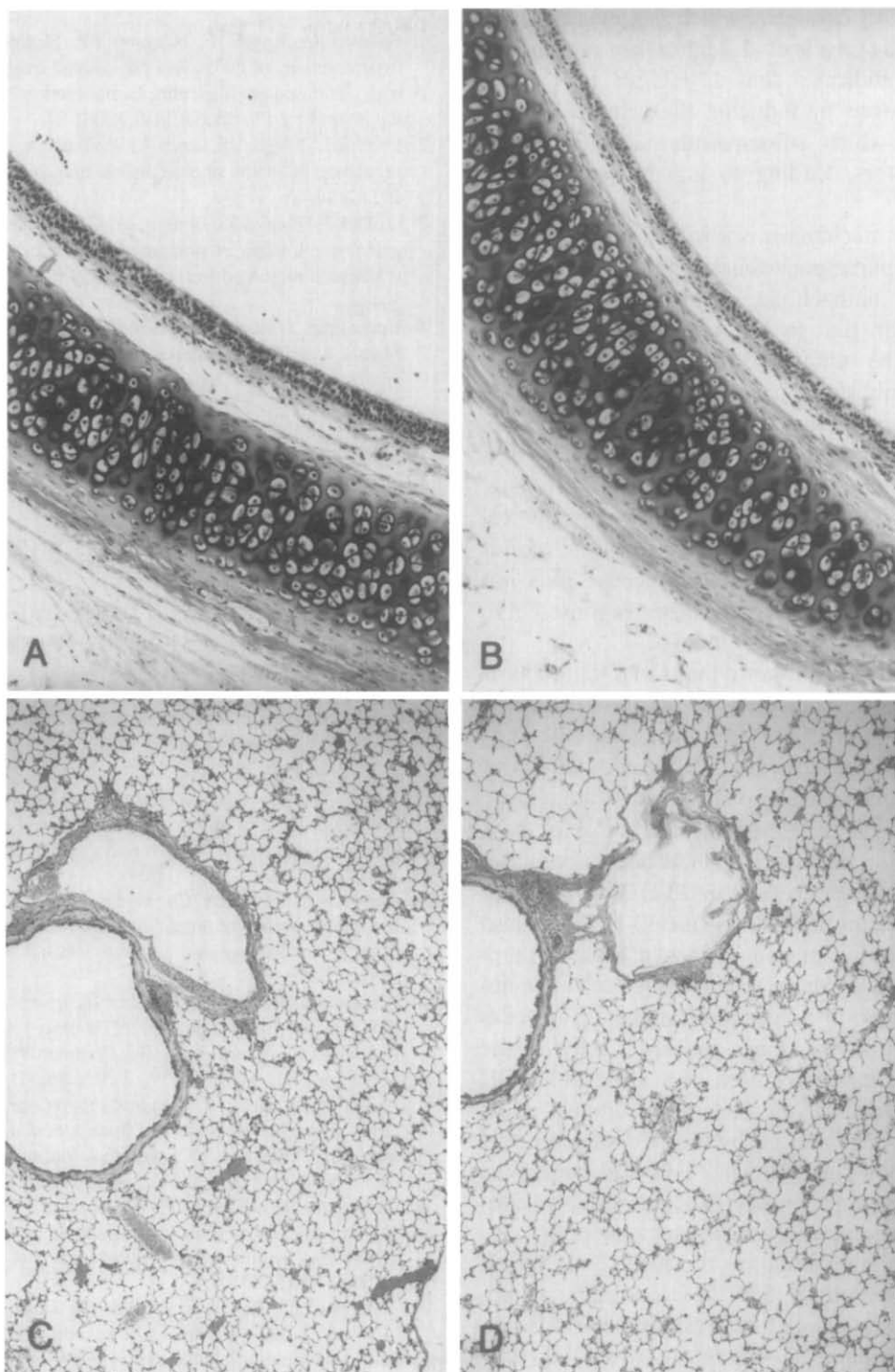


**FIG. 4.** Typical examples of histologic specimens of upper (**A, B**) and lower (**C, D**) parts of lungs from vehicle-treated animals (**A** and **C**) and 13-HODE-treated guinea pigs (**B** and **D**). Infiltration of inflammatory cells was very limited and did not differ between control and 13-HODE-treated animals. (Hematoxylin-eosin stain;  $\times 150$ .)

present there is no evidence in the literature about an increased release of mediators induced by 13-HODE other than a stimulation of the production of 12-hydroxy-arachidonic acid in platelets<sup>19</sup> and

prostacyclin in endothelial cells.<sup>21, 22</sup> However, in vitro it is possible to prevent 13-HODE-induced airway hyperresponsiveness to histamine with the neurokinin receptor antagonist FK 224 or by pre-

treatment with capsaicin, which depletes the sensory neurons (Engels et al. Manuscript in preparation). This indicates that 13-HODE may affect sensory neurons by inducing the release of neuropeptides, which subsequently activate neurokinin receptors, leading to airway hyperresponsiveness.

Whatever mechanism is involved in 13-HODE-induced hyperresponsiveness, the hyperresponsiveness to methacholine seems to be less pronounced than that to histamine (Figs. 1 and 2). However, the difference before and after 13-HODE administration with 2.0  $\mu$ g histamine is similar to the difference with 1.2  $\mu$ g methacholine. Results are presented as increase in response to a certain amount of histamine or methacholine. Pharmacologic parameters, such as maximal response and median effective concentration, cannot be calculated from these data because it is not possible to make a complete dose-response curve to these contractile agents *in vivo*.

Because it has been shown that 13-HODE induces a chemotactic response toward polymorphonuclear leukocytes,<sup>18</sup> products released from these cells (e.g., platelet activating factor and LTs) could be responsible for the increased responsiveness of the airways to contractile stimuli. However, a direct role for these inflammatory products in the induction of bronchial hyperresponsiveness caused by 13-HODE in our *in vivo* model seems unlikely because 13-HODE is also able to induce hyperresponsiveness in tracheal preparations *in vitro* where inflammatory cells are absent.<sup>14,30</sup> Moreover, no increase in inflammatory cells was observed in the upper and lower parts of the pulmonary system in guinea pigs after 13-HODE treatment (Fig. 4). Nevertheless, recruitment of inflammatory cells induced by the release of linoleic acid metabolites by epithelial cells and pulmonary macrophages may amplify the induced bronchial hyperresponsiveness *in vivo* at later time points.

In particular, eosinophils produce 13-HODE in significant amounts,<sup>31</sup> and these cells play an important role in airway hyperresponsiveness associated with asthma.<sup>32-35</sup> Therefore 13-HODE may play an important role in the inflammatory component of chronic lung diseases and the induction of bronchial hyperresponsiveness, besides the well known eosinophil-derived mediators platelet activating factor and LTC<sub>4</sub>.

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