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Natural ozone scavenger prevents asthma in sensitized rats

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Abstract—The assumption that ozone is not only a strong oxidant, but also an important inflammatory mediator, is heavily supported by the ample literature on the pulmonary toxicity and biological effects of environmental ozone and by the recent discovery that antibodies, human neutrophils, and inflammatory lesions catalyze the formation of ozone in vivo. We hypothesized that the pulmonary inflammation in asthma involves a vicious circle of ozone production and recruitment of white blood cells, which produce more ozone. Accordingly, we predicted that electron-rich olefins, which are known ozone scavengers, could be used for prophylactic treatment of asthma. In particular, volatile, unsaturated monoterpenes, could saturate the pulmonary membranes and thereby equip the airways with local chemical protection against either exogenous or endogenous ozone. Here we present experimental evidence using a sensitized rat model to support this hypothesis. Examination of the pulmonary function of sensitized rats that inhaled either limonene (unsaturated, ozone scavenger) or eucalyptol (saturated, inert to ozone) showed that limonene inhalation significantly prevents bronchial obstruction while eucalyptol inhalation does not cause any effect. The anti-inflammatory effect of limonene was also evident from pathological parameters, such as diminished peribronchiolar and perivascular inflammatory infiltrates.

1. Introduction

Asthma is a clinical syndrome that consists of recurrent episodes of wheezing, breathlessness, chest tightness, and cough associated with evidence of reversible airway obstruction and bronchial hyper-responsiveness.[1,2] The cause of asthma remains unknown;[3] however, recurrent acute and chronic inflammation has become the dominant hypothesis explaining the abnormal behavior of the airways. Various inflammatory mediators, including cytokines and chemokines, which are released from resident lung cells as well as T cells and other inflammatory cells that are recruited to the airway, influence the airway inflammation in asthma. Acute inflammation, often linked to IgE-mediated degranulation of mast cells, is thought to cause increased microvascular permeability,[4] disruption of the epithelium, and stimulation of neural reflexes and mucus-secreting glands.[5] Chronic inflammation may result in airway remodeling, characterized by hypertrophy of smooth muscle, mucus glands, and goblet cells as well as subepithelial fibrosis.

Ozone has not been considered a biological product prior to the recent report that antibodies, regardless of their antigen specificity, catalyze the conversion of singlet oxygen to an oxidant that exhibits the chemical signature of ozone.[6] These findings, and particularly the indications that ozone is also formed by human neutrophils and by inflammatory lesions, have suggested that ozone is generated, not only via the antibody-mediated water oxidation pathway,[7,8] but also by antibody-coated activated neutrophils. The involvement of ozone in inflammation has been further supported by the oxidation of 4-vinyl benzoic acid to 4-carboxybenzaldehyde by activated neutrophils[9] and by the ozonolysis of cholesterol in atherosclerotic tissues.[10,11] Furthermore, it has been proposed that since the oxidative damage of ozone would be localized to the inflammation site, this

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oxidant could be a proficient effector molecule of the immune response, which not only kills, but also functions as a signaling device that serves to amplify the inflammatory response, by the production of proinflammatory extracellular and intracellular signals.[6]

Ozone can be recognized not only by its chemical signature, but also by its unique biological effects. Numerous studies on the pulmonary toxicity of environmental ozone, a notoriously known component of air pollution, have demonstrated that exposure to ozone, even at levels below the present National Ambient Air Quality Standard, induces airway inflammation and lung injury in both humans and animals.[12] The ozone-induced pulmonary inflammation has been shown to be mediator related. Significant lung neutrophilia and increased levels of proinflammatory mediators, such as interleukin (IL)-6, IL-8, and leukotriene have consistently been found in bronchoalveolar lavage fluid (BALF) and in bronchial mucosal biopsies of healthy humans exposed to low levels of ozone, and these effects were more severe in asthmatic patients.[13–15] It has also been demonstrated that the sensory neuropeptides tachykinins, such as substance P and neurokinin A, which are present in neurons and inflammatory cells in the airway, are released by various stimuli, including ozone.[16] Exposure to ozone is known to increase exacerbations of asthma. For example, mast cells are activated and undergo degranulation in vivo upon exposure to ozone, leading to polymorphonuclear cell infiltration into the pulmonary parenchyma.[17] Moreover, it has been demonstrated that ozone is a modulator of the T-cell effector response that could account for the increased prevalence of allergic diseases in recent decades.[18]

Altogether, these representative examples and many other studies, which delineate the biological signature of ozone, strongly support the notion that ozone is not only a strong oxidant, but also an important inflammatory mediator. It has already been proposed that reactive oxygen species could act as inflammatory mediators and that asthma could involve a recurrent cycle of recruitment and stimulation of inflammatory cells, leading to the continued release of reactive oxygen species. Those predictions, however, which were made before the recent observations,[6] considered various oxidants, such as superoxide ion, hydrogen peroxide, hydroxyl radical, and hypohalous acids, but not ozone.[19] Alternatively, as has been proposed by Pryor and co-workers, ozone could be an indirect mediator, with specific lipid oxidation products (LOP), obtained during ozonolysis of unsaturated fatty acids, causing the release of known proinflammatory mediators, including platelet activation factor (PAF), IL-6, and IL-8.[20,21]

We assumed that inflammation in asthma could involve formation of ozone not only by neutrophils, but also by other white blood cells, and that ozone itself could recruit and activate more white blood cells, which, in turn, would produce more ozone. A vicious circle comprised of white blood cells recruitment and ozone production, could account for the fact that inflammation in asthma is persistent. Following this assumption, we hypothesized that electron-rich olefins, which are known to react readily with ozone, could be used for prophylactic treatment of asthma. Inhalation of volatile, hydrophobic olefins, such as unsaturated monoterpenes, could saturate the pulmonary membranes and thereby equip the airways with local ozone scavenging capability, providing chemical protection against either exogenous or endogenous ozone. Here we present experimental evidence that is based on an animal model to support this hypothesis.

2. Materials and methods

2.1. General

BN rats were obtained from Harlan Inc., USA. All experimental animals in this study were used under a protocol approved by the Institutional Animal Care and Use Committee of the Hadassah Medical School of the Hebrew University of Jerusalem. Both D-(+)-limonene (W26330-3) and eucalyptol (W24650-6) were purchased from Aldrich.

2.2. Animal sensitization

The rats were maintained on ovalbumin (OVA)-free diets. Sensitization was carried out by subcutaneous injection of 1 mg OVA (Sigma) and 200 mg of aluminum hydroxide (AlumInject, Pierce Chemical) in 0.9% (w/w) saline in a total volume of 1 mL, and intraperitoneal injection of 1 mL saline containing *Bordetella pertussis* (6×10^9 heat killed organisms) (Pasteur Marieux, France). Inhalations for 10 min each were performed with the rats, which were placed unrestrained in a 20 L box connected to an ultrasonic nebulizer (LS 230 System, France).

2.3. Inhalation of essential oils

An electric scented oil warmer loaded with either limonene or eucalyptol was placed inside the cage, producing 125 ppm of the essential oil in air, and operated continuously over a week between day 14 and day 21.

2.4. Pulmonary function test

Bronchoconstriction was measured by a modified noninvasive method, as described by Hamelmann et al.,[22] using barometric whole-body plethysmography and expressed as the enhanced pause (Penh), a calculated dimensionless value that correlates with measurement of airway resistance, impedance, and intrapleural pressure. Penh = (PEF/PIF)·((Te − Tr)/Tr) where PEP = peak expiratory flow, PIP = peak inspiratory flow, Te = expiratory time, Tr = relaxation time (time of the pressure decay to 36% of total box pressure during expiration). On day 21 post induction, Penh was measured 5 min after sensitization and airway challenge with OVA. The unrestrained, conscious rats were placed in a whole-body plethysmograph (Buxco Electronics Inc., USA) connected to a pneumotach (EMKA Technologies, Type 0000), which was connected to a 10 mL bottle

on the other end. The pneumotach was connected to a preamplifier (model MAX2270, Buxco Electronics). Analog signals from the amplifier were converted to a digital signal by an AD card and analyzed by a software package (Data Translation, USA) to calculate the respiratory rate, Te, Ti, and Penh. Statistical analytical treatment of the data was carried out using the MS Excel spreadsheet. Analysis of variance was performed on the interval data (Penh) and Kruskal Wallis Test was used for the pathology score. All error bars represent standard errors.

2.5. Pathology

On day 21 all rats were anesthetized with pentothal and sacrificed by bleeding from the abdominal aorta. Lungs were removed and inflated with 4% buffered formaldehyde under pressure of 20 cm H_2O. The lungs were sliced longitudinally and embedded in paraffin. Histologic sections 5 μm thick were cut and stained with hematoxylin and eosin. The morphological changes were evaluated by light microscopy by a pathologist who was blinded to the treatment groups. The intensity of the peribronchiolar and perivascular cellular infiltration was assessed semi-quantitatively on a 0–3 scale as follows: 0 = no or practically no inflammatory cells; 1 = a narrow rim of inflammatory cells surrounding most of the bronchioles/blood vessels, best visualized under high power; 2 = a rim of inflammatory cells 3–4 cells thick, surrounding most of the bronchioles/blood vessels; 3 = a prominent rim of inflammatory cells, 5 or more cells thick, surrounding most of the bronchioles/blood vessels. Bronchiolar constriction was also assessed on a semi-quantitative scale ranging over 0–3 grades (0 = none; 1 = mild; 2 = moderate; 3 = marked).

3. Results and discussion

Two closely related monoterpenes, limonene, **1**, and eucalyptol, **2** (Fig. 1), were examined for their ability to prevent asthmatic symptoms in sensitized rats. Both **1** and **2** share the same boiling point and a low molecular weight (136 and 154 Da, respectively). The two double bonds in **1** render the molecule a highly reactive ozone scavenger, while **2**, which is a saturated compound, is totally inert toward ozone. At first glance, tetrahydrolimonene seemed to be a more appropriate compound than eucalyptol for the control experiments. However, the physical properties of eucalyptol are closer to those of limonene [compound; vapor pressure (Torr at 25 °C); enthalpy of vaporization (KJ/mol); boiling point (°C)]: limonene, eucalyptol, tetrahydrolimonene; 1.54, 1.65, 2.16; 39.5, 39.4, 38.8; 176, 176, 168. More importantly, in view of a recent claim that eucalyptol has an anti-inflammatory activity in bronchial asthma[24] we found it interesting to use this compound for our control experiments.

3.1. Pulmonary function

Brown Norway (BN, 4-week-old) male rats were used in this study. Asthma was induced on day 0 in three groups, 10 animals each, by sensitization with ovalbumin (OVA) and aluminum hydroxide.[23] A fourth group of 10 animals was not sensitized and used as the naive control group. The three groups of sensitized rats as well as the group of naive animals were challenged every other day from day 14 until day 21 with repeated allergen (OVA) inhalation. During this period two groups of sensitized rats were continuously breathing limonene or eucalyptol, respectively.

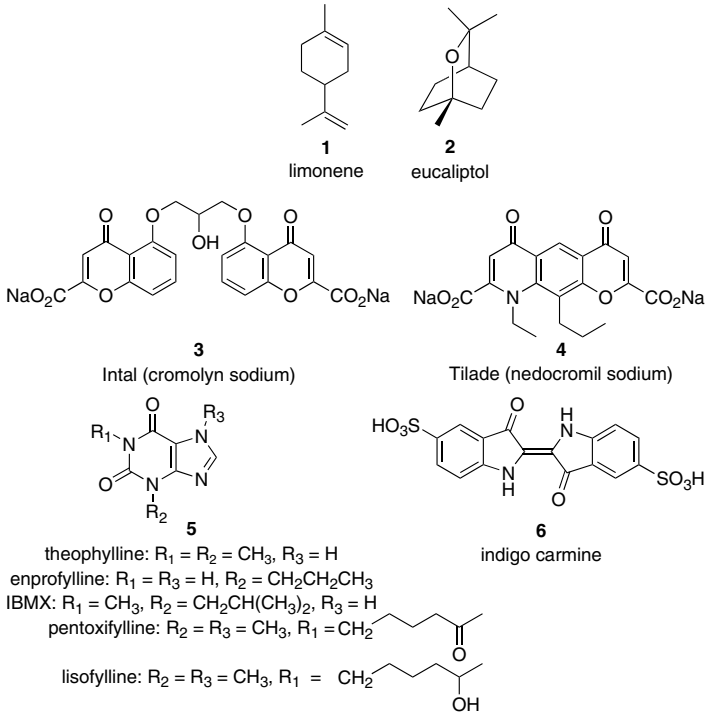

Figure 1. Ozone scavengers.

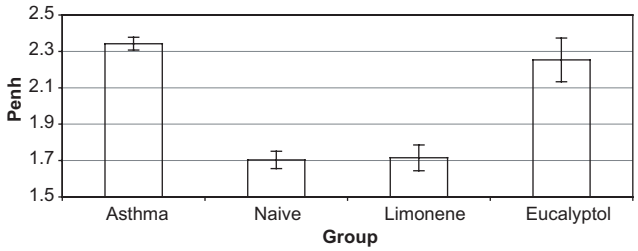

Figure 2. Results of pulmonary function test.

The Penh values (Fig. 2) of the sensitized control group (asthma) were found to be as high as those of the eucalyptol-treated sensitized rats (2.342 ± 0.004 and 2.25 ± 0.12, respectively). In contrast, the naive and the limonene-treated sensitized rats exhibited significantly lower Penh values (1.70 ± 0.05 and 1.71 ± 0.07, respectively, $p < 0.05$). These observations clearly show that limonene inhalation significantly prevented bronchial obstruction while eucalyptol inhalation did not cause any change of bronchoconstriction in the sensitized rats.

3.2. Pathology

The histologic sections included bronchioles (defined as airways lined by mucosa and lacking cartilage) and distal airways (alveolar ducts and sacs). The features examined included peribronchiolar and perivascular inflammation, morphological evidence of bronchoconstriction (papillary infolding of the bronchiolar mucosa), and granulomatous response in the pulmonary parenchyma.

Peribronchiolar and perivascular inflammatory infiltrates were composed principally of eosinophils and lymphocytes (Fig. 3). The eosinophils were disposed in a circumferential manner around the bronchioles and vessels while the lymphocytes were either similarly disposed or arranged in primary follicles (lymphoid follicles without germinal centers). In addition, multinucleated giant cells and granulomas were seen in the pulmonary parenchyma in all four groups of rats. Polarizing microscopy did not reveal foreign material. This appearance of granulomatous lesions in this asthma model was also described by Michielsen et al.[25]

Figure 3 exhibits representative examples and Figure 4 summarizes the average score of all four pathological parameters in the four experimental groups. It is clear that the relative magnitude of all parameters follow and confirm the general trend that was exhibited by the pulmonary function test (Fig. 2). Yet, there are subtle variations that may lead to further conclusions. Since the essential oils were administered by inhalation, it is expected that the bronchi would be influenced much more than the parenchyma. Indeed, as can be seen from Figure 4, rats treated with limonene showed significant

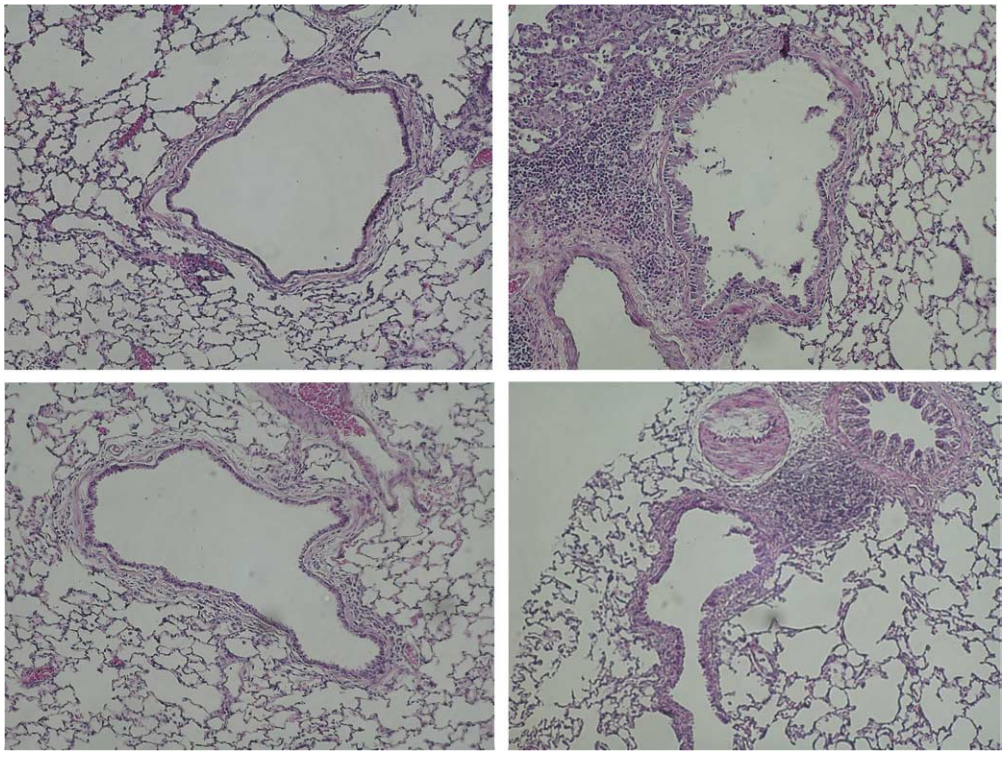

Figure 3. Pathological changes in the lungs of representative members of the four experimental BN groups. H&E original magnification × 20. Upper left: a naïve rat lung showing minimal peribronchiolar inflammatory infiltrate. Upper right: a sensitized untreated rat lung exhibiting marked inflammatory infiltrate surrounding the bronchiole in the center and evidence of bronchoconstriction (the mucosa is thrown into folds). Lower left: a limonene treated rat lung showing minimal to mild peribronchiolar inflammatory infiltration and no morphologic evidence of bronchoconstriction. Lower right: a eucalyptol treated rat lung exhibiting moderate peribronchiolar inflammation and a lymphoid follicle. The bronchiole in the upper right corner of this slide shows morphologic evidence of bronchoconstriction.

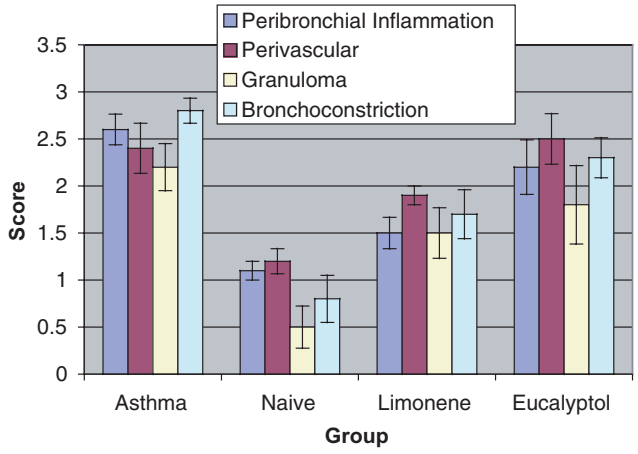

Figure 4. Results of pathological examination. All error bars represent standard errors.

reduction of peribronchial inflammatory cell infiltration in comparison with the eucalyptol-treated group (limonene 1.5 ± 0.17, eucalyptol 2.2 ± 0.29, $p < 0.05$) and, to a lesser extent, perivascular inflammatory cell infiltration (limonene 1.9 ± 0.1, eucalyptol 2.5 ± 0.27, $p < 0.05$). The morphological evidence of bronchoconstriction followed the same trend (limonene 1.7 ± 0.26, eucalyptol 2.3 ± 0.21, $p < 0.05$). In contrast, the effect on the granulomas, which are located further away from the bronchi, was found to be insignificant (limonene 1.5 ± 0.27, eucalyptol 1.8 ± 0.42, $p = 0.43$).

Limonene is one of many naturally occurring, volatile monoterpenes that contain highly substituted double bonds, which are efficient ozone scavengers.[26] Another potent ozone scavenger is isoprene,[27,28] which is one of the most abundant hydrocarbons naturally emitted by the terrestrial biosphere.[29,30] Ethylene, which is a plant ripening and senescence hormone,[31] although less reactive toward ozone than the alkyl-substituted olefins, is also emitted by the biosphere in copious quantities. These low molecular-weight lipophylic olefins can easily saturate the bronchial and alveolar membranes, thus providing chemical protection against either exogenous or endogenous ozone. One may expect that such anti-inflammatory protection would be more significant in rural rather than in urban population. It is remarkable that although rural environments are rich in various allergens that can trigger asthma, the prevalence of asthma in rural population is significantly lower in comparison with the urban incidence.[32] The increased worldwide urbanization, which is often associated not only with decreased exposure to volatile natural products, but also with more efficiently insulated and air-controlled housing, could account for the ever-increasing frequency of asthma.

Various nonnatural electron-rich olefins could also be employed for prophylactic treatment of asthma. In fact, a number of efficient ozone scavengers, particularly the various chromone, **3**, **4**, and xanthine, **5**, derivatives have long been used in the clinic for the prevention of asthma. Cromolyn sodium (CS, **3**, Intal®), which was launched in 1968, and nedocromil sodium (NS, **4**, Tilade®), have resulted from a structure–activity relationship study that was based on the natural lead compound khellin.[33,34] The latter and other naturally occurring ozone scavenging chromones are ubiquitously found in plants that have been traditionally used in folklore medicine for the treatment of asthma.[35,36] The electronic nature of the double bonds in both CS and NS, which are highly reactive toward ozone, is reminiscent of the double bond in indigo carmine, **6**, which has been widely used as a sensitive probe for quantitative detection of ozone in aqueous media.[37]

Although both CS and NS have been used in the clinic for over four decades, their mechanism of action remains unclear and neither a relevant receptor nor an endogenous ligand for these compounds has been found. Both drugs exhibit multiple pharmacologic activities.[38] CS, for example, is known to inhibit almost any component in the inflammatory cascade of events, including the IgE-mediated release of primary inflammatory mediators by mast cells and macrophages.[39,40] the accumulation and activation of eosinophils, neutrophils, and lymphocytes, the responsiveness of these cells to cytokinins and chemotaxins, the release of tachykinins, such as substance P, and so forth. We propose that the ozone scavenging capability of both CS an NS could account for much of their anti-inflammatory effect.

A number of xanthine derivatives, including theophylline, enprofylline, pentoxifylline, lisofylline, and IBMX, **5a**–**e**, have also been used in the clinic to treat asthma, although their mechanism of action is as yet unclear. For example, it has been stated that enprofylline is a poor antagonist in most classes of adenosine receptors while retaining bronchodilator potency greater than that of theophylline.[41] Chemical and medical studies have highlighted the general antioxidant properties of these xanthine derivatives.[42,43] Since the central double bond in these compounds is highly electron rich, it is likely that the pharmaceutical aptitude of **5a**–**c** involves, at least partially, ozone-scavenging capability.

In conclusion, both the pulmonary function tests and pathological data support the hypothesis that the inflammatory cascade of asthmatic events, which may involve a vicious circle of ozone production by white blood cells and recruitment of more such cells, can be intercepted by ozone scavengers. The anti-inflammatory protection provided by limonene to the sensitized animals suggests that a new pharmaceutical paradigm of controlling inflammatory diseases should be seriously considered on the basis of appropriately designed ozone scavengers. Further experiments with both animal models and human subjects are currently underway in our laboratories.

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