Bioorganic & Medicinal Chemistry Letters 25 (2015) 342–346

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl

Prophylactic treatment of asthma by an ozone scavenger in a mouse model

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ARTICLE INFO

Article history:
Received 25 August 2014
Revised 10 November 2014
Accepted 12 November 2014
Available online 20 November 2014

Keywords:
Aldehyde biomarkers
Asthma
Respiratory inflammation
D-Limonene
Systemic oxidative stress

ABSTRACT

Our hypothesis that inflammation in asthma involves production of ozone by white blood cells and that ozone could be an inflammatory mediator suggests that scavengers of reactive oxygen species (ROS), for example, electron-rich olefins, could serve for prophylactic treatment of asthma. Olefins could provide chemical protection against either exogenous or endogenous ozone and other ROS. BALB/c mice pre-treated by inhalation of D-limonene before an ovalbumin challenge exhibited significant attenuation of the allergic asthma symptoms. Diminution of the inflammatory process was evident by reduced levels of aldehydes, reduced counts of neutrophils in the BAL fluid and by histological tests. A surprising systemic effect was observed by decreased levels of aldehydes in the spleen, suggesting that the examination of tissues and organs that are remote from the inflammation foci could provide valuable information on the distribution of the oxidative stress and may serve as guide for targeted treatment.

The worldwide burden of asthma is heavy because the disease can be therapeutically alleviated but cannot be completely cured,[1] resulting in over 300 million patients and 250,000 annual deaths.[2] Therefore, efficient prevention strategies are highly desirable.[3] 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 inflammation in asthma. Chronic inflammation may result in airway remodeling, characterized by hypertrophy of smooth muscles, mucus glands, and goblet cells, as well as subepithelial fibrosis.[4–6]

Oxidative stress (OS) has been shown to play an important role in the pathogenesis of acute and chronic lung inflammatory diseases, such as asthma, lung fibrosis, and chronic obstructive pulmonary disease (COPD).[7] Increasing evidence suggests that during OS excess reactive oxygen species and reactive nitrogen species (ROS and RNS, respectively) cause imbalance between oxidants and antioxidants implicated in cellular damage.[8] For example, epithelial cells of the respiratory tract respond to different oxidant insults by selective induction of certain antioxidant enzymes.[9] However, antioxidant defense is much less effective in the airways of asthmatic patients.[10,11] Following the known observation that increased oxidative stress and decreased superoxide dismutase (SOD) activity in the asthmatic airway correlate to airflow limitation and hyper-reactivity, it has been shown that greater oxidant stress in asthma leads to greater inactivation of SOD, which likely amplifies inflammation and progressive airflow obstruction.[12] The generation of such activated species by inflammatory cells is a major microbiocidal mechanism that follows oxidative damage of cellular macromolecules, in particular DNA, proteins and lipids.[13] Certain oxidants are also endogenous signaling molecules and may also mediate important components of the inflammatory response.[14]

The notion that reactive oxygen species could act as inflammatory mediators and that asthma could involve recurrent cycles of recruitment and stimulation of inflammatory cells, is not new.[15] It has already been proposed that such cycles could lead to the continued release of reactive oxygen species, including superoxide ion, hydrogen peroxide, hydroxyl radical, and hypohalous acids. We have added ozone to this list,[16] assuming that inflammation in asthma could involve production of ozone not only by neutrophils, but also by other white blood cells, and that ozone itself could

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http://dx.doi.org/10.1016/j.bmcl.2014.11.035
0960-894X/

serve as an inflammatory mediator. A vicious circle that involves recruitment of white blood cells by ozone followed by enhance production of more ozone, 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. Consequently, inhalation of volatile, hydrophobic olefins, such as unsaturated monoterpenes, could saturate the pulmonary membranes and thereby act as local ozone scavengers and provide chemical protection against either exogenous or endogenous ozone. It has been reported that antioxidants can afford protection against virally induced inflammatory diseases.[17,18] For example, it has been demonstrated that lung pulmonary inflammation induced by respiratory syncytial virus (RSV) can be alleviated by administration of butylated hydroxy anisole, which is a known radical scavenging antioxidant.[19]

In addition to the idea that ozone itself could serve as an inflammatory mediator, it has been proposed that ozone could be an indirect mediator. Specific ozonolysis products could cause the release of known proinflammatory mediators, including platelet activation factor (PAF) and interleukins, such as IL-6, and IL-8.[20,21] It has already been demonstrated that pulmonary inflammation induced by exogenous ozone is mediator related.[22–24] Significant lung neutrophilia and increased levels of IL-6, IL-8 and leukotriene have consistently been found in BAL fluid and in bronchial mucosal biopsies of healthy humans exposed to low levels of ozone. These effects were more severe in asthmatic patients. 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.[25] Many other studies, which portray the biological signature of ozone, strongly support the notion that ozone is not only a strong oxidant, but also an important inflammatory mediator.

We have previously demonstrated that electron-rich olefins, such as D-limonene, which are known ozone scavengers, could be used for prophylactic treatment of allergic asthma in a sensitized rat model.[16] The anti-inflammatory effect of inhaled D-limonene was manifested by both pulmonary function and pathological examination. Here we show that ovalbumin-sensitized BALB/c mice, which were pretreated by inhalation of D-limonene before the ovalbumin challenge, exhibit significant attenuation of the disease symptoms. Diminution of the inflammatory process is evident by multiple indicators. Furthermore, we show here that beyond the local effect of D-limonene on lung tissues, it causes systemic effects, as reflected by decreased levels of inflammation markers in the spleen.

Using ovalbumin-sensitized BALB/c mice as an animal model of allergic asthma we studied the effect of prolonged exposure (30 days) to D-limonene. In agreement with our previous observations with the Brown Norway rat model,[16] here also, the ameliorating effect of limonene treatment was evident from multiple indicators. Attenuation of the inflammatory process in the mice model was manifested by (a) reduced production of aldehydes in all tissues, (b) reduced count of neutrophils in the BAL fluid and (c) reduced peribronchiolar inflammatory infiltration.

Oxidative stress in respiratory airway[26] can be assessed and monitored by determination of the levels of oxidants, such as hydrogen peroxide,[27] and oxidation products, such as aldehydes,[28–30] isoprostanes[31] and protein carbonyl groups.[32,33] If the oxidized biomarkers have sufficiently long lifetime they can diffuse from their membranal origin to distant intracellular or extracellular regions. In that case they can be found in various sites, such as serum plasma,[34] the BAL fluid,[35] sputum[36] and exhaled breath condensate (EBC).[37] Indeed, systemic levels of oxidized biomarkers have been observed in different matrices, such as serum plasma or EBC samples taken from asthma patients.[38–40]

Aldehydes represent one of the oxidative cleavage products of unsaturated fatty acids and their derivatives by either ozone or by various reactive oxygen species (ROS), including oxygen radicals.[41–43] For example, oxidative cleavage of oleic and palmitoleic acids produces *n*-nonanal and *n*-heptanal, respectively whereas oxidation of either arachidonic or linoleic acid yields *n*-hexanal (Scheme 1). Therefore, measuring the levels of these aldehydes in the BAL fluid could be used for monitoring airway inflammation.

Consequently, determination of malondialdehyde, *n*-hexanal, *n*-heptanal, *n*-nonanal, 4-hydroxynonenal (HNE) and 4-hydroxyhexenal (HHE) has become an efficient tool for quantitative assessment of oxidative stress. These aldehydes are considered to be byproducts of lipid peroxidation, reflecting oxidant-induced damage on unsaturated lipids in cell membranes.[44,45] The most commonly used procedure for quantitative determination of these aldehydes involved chemical derivatization prior to quantitative analysis by HPLC,[46,47] liquid chromatography-tandem mass spectrometry (LC/MS),[40,48] solid-phase micro extraction (SPME) and GC/MS.[49] Derivatization by chemical reactions, however, add significant uncertainty due to differential reaction kinetics, different yields of the various aldehydes[50,51] and cross-contamination between different sources, such as EBC, saliva, etc.[52,53]

We developed a more efficient and sensitive procedure for the quantitative assessment of oxidative stress, which does not involve any chemical modification of the aldehyde biomarkers. Direct sampling extraction of BAL fluid and tissues of lung and spleen was followed by pre-concentration prior to the quantitative GC/MS analysis. Thus, the measured levels of hexanal, heptanal and nonanal provided a quantitative indication of the severity of various lung inflammatory diseases and a direct chemical signature of an oxidative stress involving ozone and other strong oxidants.

Since the relative composition of unsaturated fatty acid derivatives varies significantly from one tissue to another we expected to measure different aldehyde profiles in the BAL fluid, lung and spleen tissues. It has already been suggested that the lung surfactant, which resides at the air/liquid interface of the pulmonary alveoli, does not only lower the surface tension but also assists with early defense against both endogenous and exogenous oxidants. Extracellular surfactants, which can be harvested from the lungs by BAL fluid, comprise mainly (90%) lipids. Most of the latter are phospholipids (80–90%) in addition to small amounts of neutral lipids, such as cholesterol.[54] Phosphatidylcholin (PC) represents the main constituent of the phospholipids (50–60%) and its major component is dipalmitoyl phosphatidylcholin (68%) as well as mono-palmitoyl mono-unsaturated fatty acids. For example, analysis of the PC fraction in rat lungs without differentiation between tissue and alveolar fluid indicates that it contains oleic, palmitoleic, linoleic and arachidonic acids at 6.4%, 10.1%, 5.4% and 3.1%, respectively.[55] In contrast, analysis of the PC fraction in spleen tissue reveals a remarkably low level of palmitoleic acid (<0.2%) and high levels of oleic and arachidonic acids (13.3% and 9%, respectively). The level of linoleic acid was found to be low in both tissues.[56]

We monitored the aldehyde profiles of all mice groups in three different locations: BAL fluid, lung tissues and spleen tissues (Fig. 1). As can be concluded from the control experiments, the BAL fluid of healthy mice contained more oleic acid derivatives than linoleic and arachidonic acids, whereas the situation was reversed in lung and spleen tissues with linoleic and arachidonic acid derivatives being more abundant. This is reflected in small but measureable background levels of the corresponding aldehydes in unsensitized mice under normal conditions: small excess of nonanal over hexanal in the BAL fluid and excess of hexanal over nonanal in the lung and spleen tissues. All samples contained small