

Original Contribution

Reduction of antigen-induced respiratory abnormalities and airway inflammation in sensitized guinea pigs by a superoxide dismutase mimetic

Emanuela Masini^{a,*}, Daniele Bani^b, Alfredo Vannacci^a, Simone Pierpaoli^a,
Pier Francesco Mannaioni^a, Suzy A.A. Comhair^c, Weiling Xu^c,
Carolina Muscoli^{d,e}, Serpil C. Erzurum^c, Daniela Salvemini^e

^aDepartment of Preclinical and Clinical Pharmacology, University of Florence, Florence, Italy

^bDepartment of Anatomy, Histology and Forensic Medicine, Section of Histology, University of Florence, Florence, Italy

^cDepartments of Pulmonary and Critical Care Medicine, and Cancer Biology, The Lerner Research Institute, The Cleveland Clinic Foundation, Cleveland, OH 44195, USA

^dFaculty of Pharmacy, University of Catanzaro "Magna Graecia", Roccelletta di Borgia, Catanzaro, Italy

^eDepartment of Biological and Pharmacological Research, Metaphore Pharmaceuticals, 1910 Innerbelt Business Center Drive, St Louis, MO 63114, USA

Received 29 April 2004; revised 31 January 2005; accepted 5 April 2005

Available online 30 April 2005

Abstract

Reactive oxygen species have been implicated in the pathogenesis of asthma and, in atopic asthmatics, endogenous superoxide dismutase (SOD) enzyme levels are known to decrease. This suggests that replacing a failed endogenous SOD enzyme system with a mimetic of the endogenous enzyme would be beneficial and protective. In this study we demonstrate that removal of superoxide by the SOD mimetic (SODm) M40403 reduces the respiratory and histopathological lung abnormalities due to ovalbumin (OA) aerosol in a model of allergic asthma-like reaction in sensitized guinea pigs. Both respiratory abnormalities and bronchoconstriction in response to OA challenge are nearly absent in naïve animals, while they sharply became severe in sensitized animals. In addition, OA aerosol induced a reduction of MnSOD activity which was paralleled with bronchiolar lumen reduction, pulmonary air space hyperinflation, mast cell degranulation, eosinophil infiltration, bronchial epithelial cell apoptosis, increase in myeloperoxidase activity, malonyldialdehyde production and 8-hydroxy-2'-deoxyguanosine formation in the lung tissue, as well as elevation of PGD₂ in the bronchoalveolar lavage fluid. Treatment with M40403 restored the levels of MnSOD activity and significantly reduced all the above parameters. In summary, our findings support the potential therapeutic use of SOD mimetics in asthma and anaphylactic reactions and account for a critical role for superoxide in acute allergic asthma-like reaction in actively sensitized guinea pig.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Superoxide dismutase mimetic; Guinea pig; Asthma-like reaction; Superoxide; Mast cells; Eosinophils; Myeloperoxidase; Malonyldialdehyde; 8-hydroxy-2'-deoxyguanosine; PGD₂

Introduction

Asthma is defined by airway inflammation and hyper-responsiveness. Damage and loss to airway epithelium, leukocyte infiltration, mast cell degranulation, smooth muscle hyperplasia, thickening and aberrant collagen, and protein composition of the basement membrane are well-established elements of the asthmatic airway [1]. Physical loss of epithelial lining cells is considered a proximate cause of airway hyperreactivity to inhaled mediators and has been

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MBP, (eosinophilic) major basic protein; MDA, malonyldialdehyde; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; OA, ovalbumin; PAO, pressure at the airway opening; PBS, phosphate-buffered saline; PGD₂, prostaglandin D₂; SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; SODm, superoxide dismutase mimetic; TdT, terminal deoxynucleotidyl transferase.

* Corresponding author. Fax: +39 055 4271233.

E-mail address: emanuela.masini@unifi.it (E. Masini).

speculated to contribute to asthmatic airway remodeling, together with abnormal collagen synthesis. Clearly, agents that are able to protect against epithelial cell loss and inflammation could be of therapeutic use. A large number of studies indicate that increased levels of oxygen-derived free radicals and high-energy oxidants produced by inflammatory and epithelial cells in the airway are important mediators in the pathogenesis of asthma [2–4]. For example, in experimental models, reactive oxidant species can reproduce the characteristic features of asthma, including airway hyperreactivity, bronchoconstriction, β -adrenergic dysfunction, cytokine production, mucus hypersecretion, microvascular leak with edema, epithelial damage, and sloughing [2].

Direct evidence for the role of free radicals in asthma also comes from human studies that demonstrate that the level of bronchial obstruction in asthma is associated with an increased spontaneous and stimulus-induced production of oxygen free radicals in airway lumen by inflammatory cells [3]. As a reaction, the levels of antioxidants in airways of asthmatics are increased in the early phase of the disease, whereas they undergo rapid consumption during the evolution of the disease [5–7].

Several cells within the lungs have been proposed to have a pivotal role for the initiation and maintenance of airway inflammation: among them, lung mast cells play a critical role [8,9]. Besides the classical allergen-dependent pathways, reactive oxygen and nitrogen species produced in the lung during airway inflammation can also activate mast cells to release mediators such as histamine, prostaglandin D_2 (PGD₂), cysteinyl leukotrienes, and specific serine protease tryptase [9,10], which in turn exaggerate the response.

Superoxide dismutase (SOD) enzymes are a primary component of the antioxidant defense system that limits lung injury from oxygen radicals but they are prone to inactivation by superoxide radicals [11,12]. In fact, chronic asthmatic patients have reduced levels of superoxide dismutase activity in lung cells and in lung epithelial lining fluid as compared with healthy control subjects [13]. Loss of SOD activity, together with increased oxidants in the asthmatic airways, may contribute to the pathophysiology of asthma [14]. While the cause of the loss of SOD activity is not known for sure, it is possible that the enzyme is nitrated by peroxynitrite, the reaction product of superoxide with nitric oxide [15] and thereby inactivated [16–19]. The potential role of superoxide/peroxynitrite in asthma has been further supported by the recent observation that isolated bronchial preparations from transgenic mice overexpressing Cu/Zn SOD are resistant to allergen-induced constriction as compared with wild-type controls [20]. In this context, we wondered why superoxide dismutase mimetics would be an effective therapy for asthma. In this study we have evaluated the pharmacological activity of the superoxide dismutase mimetic M40403 in a well-established in vivo model of allergic asthma-like reaction in sensitized guinea pigs [21–24].

M40403 is a stable, low molecular weight, manganese-containing, nonpeptidic molecule possessing the function and catalytic rate of native superoxide dismutase enzymes, but with the advantage of being a much smaller molecule (MW 483 vs MW 30,000 for the mimetic and native enzyme, respectively) [25,26]. Furthermore, M40403 is stable in vivo, penetrates cells readily, reaches a wide tissue distribution in rats, is excreted with no detectable dissociation, and is recovered intact in urine and feces [26]. M40404, the inactive congener, has no SOD catalytic activity and is pharmacologically inactive in vivo in models of inflammation [26]. This was used as the control for M40403.

Materials and methods

Reagents

Unless otherwise specified, all substances used were obtained from Sigma–Aldrich (Dorset, UK). Ovalbumin (OA) was obtained from Fluka (Buchs, Switzerland). The SOD mimetic M40403, and its inactive isomer M40404 were synthesized from MetaPhore Pharmaceutical (St. Louis, MO). The structures of M40403 and M40404 are shown in Fig. 1.

Animals

Male Hartley albino guinea pigs were used. They were purchased from a commercial dealer (Rodentia, Bergamo,

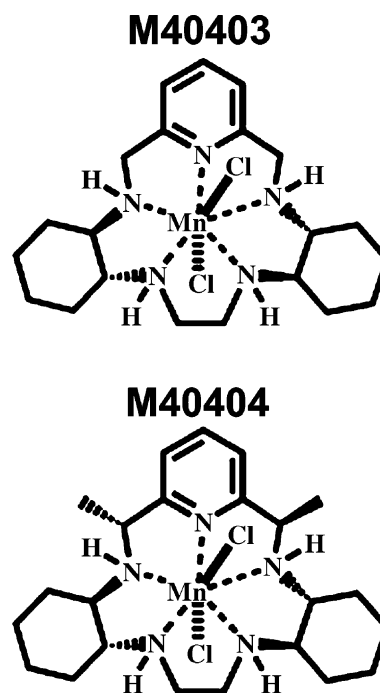


Fig. 1. Structure of the active (M40403) and inactive (M40404) superoxide dismutase mimetics used in this study.

Italy) and quarantined for 7 days at 22–24°C with a 12-h light, 12-h dark cycle before use. Standard laboratory chow (Rodentia), fresh vegetables, and water were available *ad libitum*. The experimental protocol was designed in compliance with the recommendations of the European Economic Community (86/609/CEE) for the care and use of laboratory animals and in agreement with the Good Laboratory Practice. It was approved by the animal care committee of the University of Florence (Florence, Italy). At the end of the treatment, the animals weighed 350–400 g.

Sensitization and treatments of animals

Guinea pigs were divided into 7 experimental groups. Guinea pigs of group 1 ($n = 12$) were injected with isotonic saline (5 ml/kg *ip*, plus 5 ml/kg *sc.*) and 18–21 days later, these animals were treated with an aerosol of OA in saline (5 mg/ml) and used as negative controls. They are referred to as naïve animals. The remaining guinea pigs were sensitized with 100 mg/kg *ip* plus 100 mg/kg, *sc* OA, suspended in saline (20 mg/ml). After 18–21 days, they were challenged with an aerosol of OA in saline (5 mg/ml) to verify sensitization, by evaluating the onset of allergen-elicited cough, as described below. The animals were withdrawn from antigen exposure at the first cough stroke. The guinea pigs reactive to the inhaled antigen were used for the further experiments. With the chosen protocol, only 5 animals out of 77 (6.5% of total) failed to develop sensitization. Three days later, the sensitized guinea pigs were divided in the following groups:

Group 2 ($n = 12$). Treatment with an injection of 0.5 ml of vehicle (sodium bicarbonate buffer 26 mM, pH 8.3) *ip* 6 h before undergoing OA provocation test, as described below. They are referred to as sensitized animals.

Group 3 ($n = 8$). Treatment with M40403, dissolved in the above vehicle, at the dose of 1 mg/kg *ip*, 1 h before OA provocation test.

Group 4 ($n = 8$). Treatment with M40403, as in group 3, 3 h before OA provocation test.

Group 5 ($n = 12$). Treatment with M40403, as in group 3, 6 h before OA provocation test.

Group 6 ($n = 8$). Treatment with M40403, as in group 3, 9 h before OA provocation test.

Group 7 ($n = 12$). Treatment with M40404, the inactive congener of M40403, dissolved in the vehicle at the dose of 1 mg/kg *ip*, 6 h before OA provocation test.

The dose of M40403 used in the present study is similar to that found effective in reducing inflammatory parameters in previous animal studies [27,28].

Evaluation of respiratory activity

The guinea pigs of all groups were individually placed in an airtight transparent whole-body chamber, as previously

described [21–24]. The changes in inner pressure in the respiratory chamber induced by breathing were monitored with a high-sensitivity pressure transducer (Battaglia–Rangoni, Bologna, Italy) connected to a PC2400A channel polygraph (Battaglia–Rangoni). Upon breath stabilization, usually occurring within 30–60 s, guinea pigs were challenged with an aerosol of OA (5 mg/ml in saline) for 10 s. With the used device, very small aerosol particles are obtained that can easily reach the lower respiratory airways, as assessed previously [29]. The naïve guinea pigs (group 1) were included in the aerosol challenge to reveal possible breath alterations due to nonspecific stimulation of the airways by the aerosol droplets. The respiratory activity of the animals subjected to the different treatments was monitored for 10 min after the onset of aerosol administration and classified according to the criteria reported previously [21–24]. Namely, cough was assumed as a transient change in the pressure (a rapid inspiration followed by a rapid expiration), whereas dyspnea was assumed as a series of irregular breaths of abnormal frequency (tachypnea) and amplitude or as repeated gasping. Movements of the guinea pigs were visually monitored by two trained observers, who were blinded to group assignment of the animals. In this way, any motion- and sneezing-related changes in the inner pressure of the body chamber could also be disregarded. The following parameters were evaluated [21,24]: (i) latency time (s) for the first cough stroke; (ii) cough severity, the product of cough frequency (cough strokes/min) and mean cough amplitude (excess pressure over the normal breath); (iii) latency time (s) for the onset of dyspnea. At the end of the experiments (i.e., 10 min after aerosol administration) the animals were killed by decapitation and, upon thoracotomy, the gross appearance of the lungs was examined. In 5 animals from each group, lung tissue samples from the middle and the lower lobes were taken for biochemical and morphological analyses, as described below. Before opening the thorax, 3 animals from each group were subjected to bronchoalveolar lavage by insertion of a cannula into the trachea and instillation of 3 ml of PBS, pH 7.4. Bronchi were washed 3 times before collection of bronchoalveolar fluid, which was then centrifuged at 1100g for 30 min. The cell-free supernatant was collected, and its volume was measured and frozen at –70°C until needed.

Measurement of bronchoconstriction

This was performed on anesthetized, mechanically ventilated guinea pigs by a constant volume method, as reported previously [30]. Four animals from groups 1 (naïve), 2 (sensitized, vehicle-treated), 5 (sensitized, treated with M40403 6 h before OA challenge), and 7 (sensitized, treated with M40404 6 h before OA challenge) were injected *ip* with 100 mg/kg sodium penthotal (Abbott, Latina, Italy) to induce anesthesia and abolish natural respiration. Body temperature was monitored continuously

and maintained constant at 34.5°C. The trachea was cannulated with a polyethylene tube (inner diameter, 2.1 mm) and the animals were ventilated artificially with a small-animal respirator (Harvard, Edenbridge, UK), adjusted to deliver a tidal volume of 10 ml/kg at a rate of 60 strokes/min. Tidal volume was kept constant by a water valve providing an initial basal pressure of 1.2–1.7 kPa. Changes in lung resistance to inflation (pressure at the airway opening; PAO), specifically the lateral pressure of the inlet air tube, were registered by a pressure transducer connected to a polygraph (Battaglia–Rangoni). After 10 min of stabilization, the basal inflation pressure was measured. Then, each animal was exposed to an OA aerosol for 1 min, given through the inlet air pipe. Changes in inflation pressure, which are directly related to airway resistance, were recorded for 10 min after the beginning of OA aerosol and expressed as percentage changes over the basal values.

Histological and morphometrical analyses

Lung tissue samples, 2 from each animal, were fixed by immersion in Mota fluid, dehydrated in graded ethanol, and embedded in paraffin. This fixative solution allows a rapid infiltration of the tissue, with only minimal artifactual shrinking, thus providing a tissue morphology which is representative of the lung features *in vivo*. Lung tissue sections, 5 μm thick, were used for morphometric analysis, as previously reported [24].

A first series of determinations was carried out on hematoxylin and eosin-stained sections to evaluate the surface area of alveolar air spaces. In each guinea pig, determinations were performed on tissue sections cut from the 2 different lung samples, examined with a $\times 10$ objective. Four randomly chosen microscopical fields per animal (2 fields per section) were analyzed. At the chosen magnification, each field corresponds to a tissue area of 570,224 μm^2 that includes an average of 300 alveolar profiles. The same tissue sections were used to evaluate the surface area of bronchial lumina, selected by: (i) histological appearance of small-sized, muscular bronchi; (ii) transverse or slightly oblique cross section. In each guinea pig, measurements were carried out on 4–6 randomly chosen bronchi from the tissue sections cut from the 2 different lung samples, examined with a $\times 20$ objective. For both alveolar and bronchial luminal areas, the microscopical fields to be analyzed were registered by a CCTV television camera (Model S 75D1, World Precision Instruments, Sarasota, FL) applied to a Reichert–Jung Microstar IV light microscope (Cambridge Instruments, Buffalo, NY) and interfaced with a personal computer through a Matrox Marvel G400–TV digitizing card (Matrox Graphics, Dorval, Canada). On the digitized images, surface area measurements were carried out using the Scion Image Beta 4.0.2 image analysis program (Scion Corp., Frederick, MD) upon the appropriate threshold to include only blank,

tissue-free air spaces. The mean values ($\pm\text{SE}$) of alveolar and bronchial luminal areas were then calculated for each experimental group.

A second series of determinations was carried out on sections stained with Astra blue (Fluka, Buchs, Switzerland) to evaluate the optical density of lung mast cells, which is related to the content of secretory granules. In each guinea pig, determinations were performed on tissue sections cut from the 2 different lung samples, according to the method described previously for similar purposes [31]. The mast cells were viewed by the same image analysis device described above, using a $\times 100$ oil immersion objective. In particular, the Matrox Marvel G400–TV card allows for the light transmitted across the microscopical slide to be determined within a range of 256 gray levels, which are between 0 (black level) and 255 (white level). The card also allows for a digitized image of mast cells to be reproduced on the basis of the values estimated. Measurements of optical density were carried out on selected mast cell profiles using the Scion Image Beta 4.0.2 image analysis program. In each animal, 30 different mast cells, 15 from each lung sample, were analyzed and the mean optical density value ($\pm\text{SE}$) was then calculated for the entire experimental group.

A third series of determinations was carried out on sections immunostained with anti-major basic protein (MBP), a marker for eosinophils. Sections were rehydrated, treated with 0.1% trypsin for 10 min to retrieve antigen and then with 0.3% H_2O_2 (v/v) in 60% methanol (v/v) to quench endogenous peroxidase, and, finally, incubated overnight with mouse monoclonal anti-human eosinophil (MBP) antibodies (clone BMK13, Chemicon, Temecula, CA; 1:50 in PBS). Immune reaction was revealed by the indirect immunoperoxidase method (Vectastain Elite kit, Vector, Burlingame, CA), using 3,3'-diaminobenzidine as chromogen. As negative controls, sections incubated with only the primary or the secondary antisera were used. In each guinea pig, the number of MBP-positive eosinophils was counted in 10 randomly chosen microscopical fields at a $\times 200$ final magnification (test area: 72,346 μm^2). Values obtained from two different observers were averaged.

DNA-nicking assay for apoptosis in airway epithelial cells

Loss of airway epithelial cells has been speculated to contribute to airway hyperreactivity and asthmatic airway remodeling. Furthermore, asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis [32]. Therefore, a DNA-nicking assay was carried out on conventional histological sections, upon paraffin removal and rehydration, by the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay (Boehringer Mannheim, Indianapolis, IN). This method allows detection of apoptotic cells through the labeling of DNA strand breaks by terminal deoxynucleotidyl transferase, which polymerizes fluorescein-labeled nucleotides to free

3'-OH DNA ends in a template-independent manner (TUNEL reaction). Incorporation of the labeled nucleotides was revealed using an anti-fluorescein antiserum conjugated with alkaline phosphatase and vector red alkaline phosphatase substrate K (Vector Laboratories, Burlingame, CA) or nitroblue tetrazolium (NBT)/BCIP (Roche Diagnostics, Indianapolis, IN). The percentage of positive epithelial cells was evaluated by two different observers and the values were then averaged.

Determination of lung tissue myeloperoxidase (MPO) activity

MPO activity, an indicator of leukocyte accumulation, was determined as previously described [33]. Frozen lung tissue samples of about 100 mg were homogenized in a solution containing 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7) and then centrifuged for 30 min at 20,000g at 4°C. An aliquot of the supernatant was then allowed to react with a solution of *tetra*-methylbenzidine (1.6 mM) and 0.1 mM H₂O₂. The rate of change in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 µmol of peroxide per minute at 37°C (expressed in mU/mg wet tissue).

Determination of lung tissue malonyldialdehyde (MDA) production

MDA is an end-product of peroxidation of cell membrane lipids caused by oxygen-derived free radicals and is considered a reliable marker of inflammatory tissue damage. It was determined by measurement of the chromogen obtained from the reaction of MDA with 2-thiobarbituric acid, according to Aruoma et al. [34]. About 100 mg of lung tissue were homogenized with 1 ml of 50 mM Tris-HCl buffer containing 180 mM KCl and 10 mM EDTA, final pH 7.4. Then, 0.5 ml of 2-thiobarbituric acid (1% w/v) in 50 mM NaOH and 0.5 ml of HCl (25% w/v in water) were added to 0.5 ml of sample. The mixture was placed in test tubes, sealed with screw caps, and heated in boiling water for 10 min. After cooling, the chromogen was extracted in 3 ml of 1-butanol, and the organic phase was separated by centrifugation at 2000g for 10 min. The absorbance of the organic phase was read spectrophotometrically at 532 nm wavelength. Protein concentration was determined with the Bradford method. The values are expressed as nanomoles of thiobarbituric acid-reactive substances (MDA equivalents) per milligram of protein, using a standard curve of 1,1,3,3-tetramethoxypropane.

Determination of 8-hydroxy-2'-deoxyguanosine

Frozen lung samples were thawed at room temperature and cell DNA isolation was performed according to

Lodovici et al. [35], with minor modifications. The samples were homogenized in 1 ml of 10 mM phosphate-buffered saline, pH 7.4, sonicated on ice for 1 min, added with 1 ml of 10 mM Tris-HCl buffer, pH 8, containing 10 mM EDTA, 10 mM NaCl, 0.5% SDS, and incubated for 1 h at 37°C with 20 µg/ml RNase (Sigma). Then, the samples were incubated overnight at 37°C under oxygen-free conditions by insufflating argon, in the presence of 100 µg/ml proteinase K (Sigma). After incubation, the mixture was extracted with chloroform/isoamyl alcohol (10/2 v/v). DNA was precipitated from the aqueous phase with 0.2 vol of 10 M ammonium acetate, solubilized in 200 µl of 20 mM acetate buffer, pH 5.3, and denaturated at 90°C for 3 min. The extract was then supplemented with 10 IU of P1 nuclease in 10 µl and incubated for 1 h, at 37°C with 5 IU of alkaline phosphatase in 0.4 M phosphate buffer, pH 8.8. All the procedures were performed in the dark under argon. The mixture was filtered by an Amicon Micropure-EZ filter (Amicon, MA) and 50 µl of each sample was used for 8-hydroxy-2'-deoxyguanosine (8-OHdG) determination using a Bioxytech EIA kit (Oxis, Portland, OR), following the instructions provided by the manufacturer. The values are expressed as nanogram of 8-OHdG per milligram of protein.

Measurement of MnSOD activity

The frozen lung samples were homogenized with 10 mM phosphate buffered saline, pH 7.4, sonicated on ice for 1 min and centrifuged at 100g for 10 min. Supernatants were used for SOD measurement. The assay of MnSOD activity was basically the same as described by Beauchamp and Friedovich [36], as modified by Nishida et al. [37]. The assay is based on SOD-induced inhibition of the conversion of nitroblue tetrazolium into a blue tetrazolium salt mediated by superoxide radicals generated by xanthine oxidase. The reaction was performed in sodium carbonate buffer, 50 mM, pH 10.1, containing 0.1 mM EDTA, 25 µM NBT (Sigma), 0.1 mM xanthine, and 2 nM xanthine oxidase (Boehringer). The rate of reduction of NBT was monitored with a Perkin Elmer spectrophotometer set at 560 nm. The amount required to inhibit the rate of reduction of NBT by 50% was defined as 1 unit of SOD activity. Specific MnSOD activity was calculated by inhibiting total SOD activity preincubating the sample for 30 min with 2 mM NaCN. Values are expressed as milliunits per milligram of proteins.

Determination of PGD₂ in bronchoalveolar lavage fluid

Production of PGD₂, the major cyclooxygenase product generated by activated mast cells during allergic response [38], was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical, Ann Arbor, MI), following the protocol provided by the manufacturer. Results are expressed as nanogram per millilitre of bronchoalveolar lavage fluid.

Statistical analysis

The reported data are expressed as the mean $\pm$ SE of independent experiments. Statistical analysis was performed by one-way ANOVA, followed by the Student–Newman–Keuls multiple comparison post hoc test; $P < 0.05$ was considered significant. Calculations were done using a GraphPad Prism 2.0 statistical program (GraphPad software, San Diego, CA).

Results

Reduction by M40403 of OA-induced respiratory abnormalities and bronchoconstriction

The mean values of the respiratory parameters assayed are reported in Figs. 2 and 3. There were no substantial abnormalities in the nonsensitized, naïve guinea pigs (group 1) after inhalation of OA, apart for sporadic cough strokes starting about 3–4 min after the onset of the aerosol. Challenge of sensitized, vehicle-treated guinea pigs with OA aerosol (group 2) resulted in striking abnormalities in the respiratory pattern, consisting of a significant reduction of cough latency (Fig. 2A), a significant increase in cough severity (Fig. 2B), and a significant decrease for the onset of dyspnea (Fig. 2C). Conversely, treatment of the sensitized guinea pigs with M40403 (1 mg/kg bw) 1 to 9 h before OA aerosol (groups 3–6), resulted in a clear-cut, statistically significant reduction of the grade of respiratory abnormalities as compared with the animals treated with the vehicle (group 2), or with those which received the inactive SODm M40404 in the place of authentic M40403 (group 7). In particular, pretreatment with M40403 increased cough latency (Fig. 2A), and reduced cough severity (Fig. 2B), while clear-cut episodes of dyspnea did not occur or, if any, they were markedly delayed in comparison with the sensitized animals not given M40403 (groups 2 and 7) (Fig. 2C). The maximum protective effect was observed when M40403 was administered 6 h before OA aerosol. Similarly, the experiments with mechanically ventilated animals showed that, in the sensitized, vehicle-treated guinea pigs (group 2), OA challenge induced a prompt, clear-cut increase in inflation pressure (PAO), indicating the occurrence of bronchoconstriction. This effect of OA was significantly reduced in the animals treated with M40403 6 h before the aerosol challenge (group 5), but not in those treated for the same time with the inactive enantiomer M40404 (group 7) (Fig. 3).

Reduction by M40403 of OA-induced lung morphological abnormalities

Macroscopic examination of the lungs showed prominent changes in the sensitized, vehicle-treated guinea pigs challenged with OA (group 2) compared with the naïve

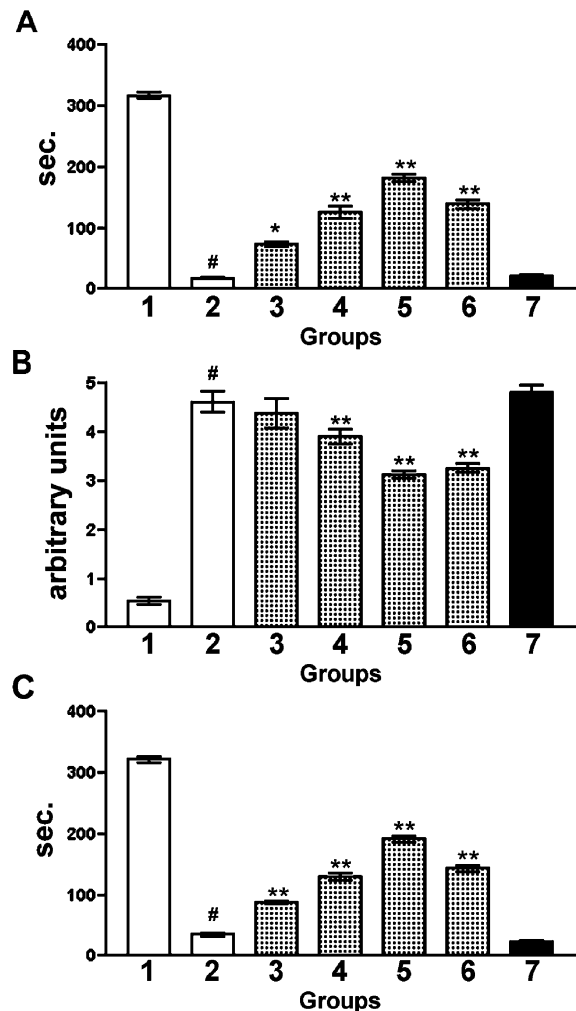


Fig. 2. Effects of M40403 (1 mg/kg bw), given 1, 3, 6, and 9 h before OA challenge (groups 3–6, dotted bars) and of its inactive enantiomer M40404 (1 mg/kg bw), given 6 h before OA challenge (group 7, black bar) on cough latency (A), cough severity (B), and latency for the onset of dyspnea (C). Data are expressed as mean $\pm$ SE of 8 experiments. [#] $P < 0.01$ vs naïve animals (group 1); ^{*} $P < 0.05$, ^{**} $P < 0.01$ vs sensitized, vehicle-treated animals (group 2).

ones (group 1), mainly consisting in marked swelling of the pulmonary lobes due to the accumulation of air. Sectioning of trachea or of main bronchi did not cause lung deflation, thus indicating that obstruction of peripheral bronchi had occurred. Lung inflation was not observed in the animals treated with M40403 (data not shown).

Light microscopy of lung tissue showed that the intrapulmonary bronchioli and respiratory air spaces of naïve guinea pigs had a normal appearance. In particular, intrapulmonary bronchioli showed open lumina, with bronchial mucosa forming short folds, and alveolar air spaces were evenly small sized (Fig. 4). Conversely, in the sensitized, vehicle-treated guinea pigs challenged with OA (group 2) we observed a reduction of the lumen of intrapulmonary bronchi and, in large areas of the lung parenchyma, a marked dilation of the alveolar air spaces due to accumulation of entrapped air (Fig. 4B). In the guinea pigs

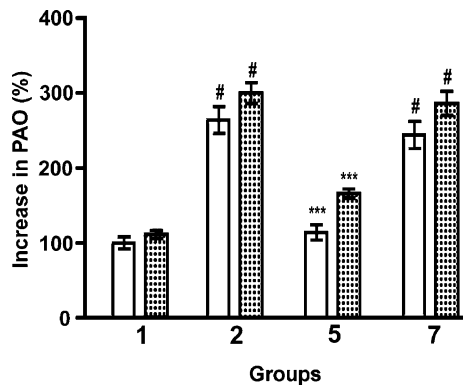


Fig. 3. Effects of pretreatment with M40403 (1 mg/kg bw) for 6 h (group 5) or with its inactive enantiomer M40404 (1 mg/kg bw) for 6 h (group 7) on OA-induced bronchoconstriction in anesthetized, mechanically ventilated guinea pigs, evaluated at 5 min (open bars) and 10 min (dotted bars) after OA aerosol. The pressure at the airway opening (PAO) is expressed as percentage changes over basal inflation pressure before OA challenge, assumed as 100%. Data are means $\pm$ SEM ($n = 4$). [#] $P < 0.001$ vs naïve animals (group 1); ^{***} $P < 0.001$ vs sensitized, vehicle-treated animals (group 2).

pretreated for 6 h with M40403 (group 5), the above lung histopathological changes were substantially reduced (Fig. 4C). In fact, the intrapulmonary bronchi did not show appreciable signs of constriction and the alveolar air spaces were not dilated. Morphometric analysis confirmed the visual observation that M40403 prevents bronchiolar constriction and pulmonary air space inflation (Fig. 5). In fact, in the guinea pigs given M40403 (group 5), the luminal area of alveolar air spaces was significantly reduced while that of

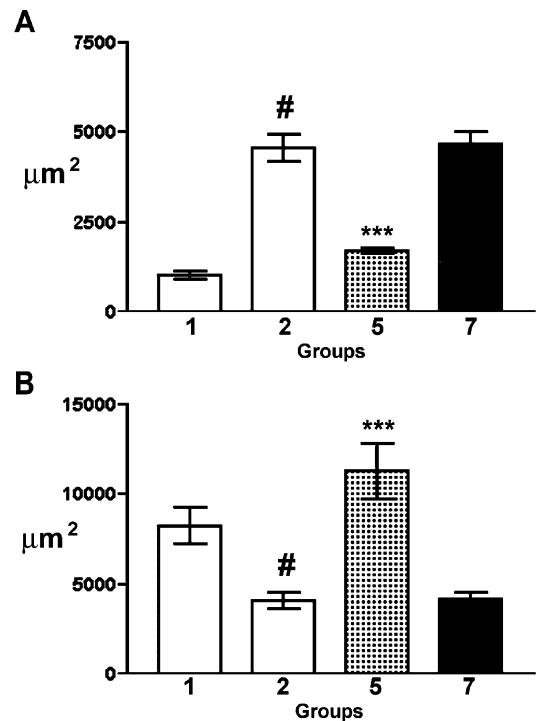


Fig. 5. Morphometric analysis of luminal area of respiratory air spaces (A) and bronchioli (B). M40403 (1 mg/kg bw; group 5) (dotted bar), but not its inactive enantiomer M40404 (1 mg/kg bw; group 7) (black bar), given 6 h before OA challenge to sensitized guinea pigs reduces bronchiolar constriction and respiratory air space dilation, which are prominent in the sensitized, vehicle-treated animals (group 2). [#] $P < 0.001$ vs naïve animals (group 1); ^{***} $P < 0.001$ vs vehicle-treated animals.

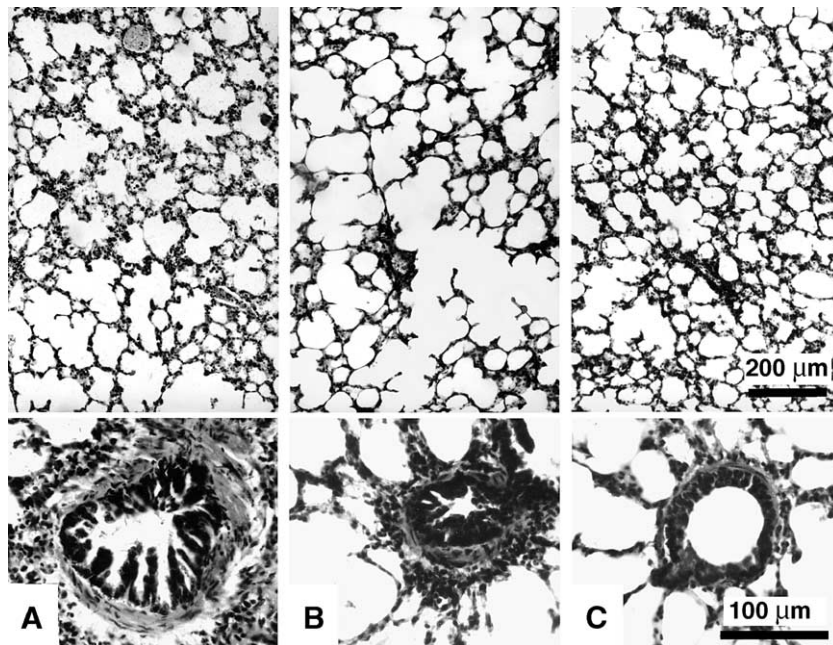


Fig. 4. Representative lung tissue micrographs from: (A) naïve guinea pigs (group 1); (B) sensitized, vehicle-treated guinea pigs (group 2); (C) sensitized guinea pigs treated with M40403 (1 mg/kg bw) 6 h before OA challenge (group 5). Compared with panel A, panel B shows a marked dilation of respiratory air spaces and reduction of bronchiolar lumen. These alterations are not present in the M40403-treated guinea pigs (C). Hematoxylin and eosin.

bronchioli was significantly increased as compared to the vehicle-treated (group 2) or the M40404-treated animals (group 7).

Reduction by M40403 of OA-induced mast cell granule release

By densitometry of Astra blue-stained mast cells (Fig. 6), we observed a significant decrease in mast cell optical density, indicating a discharge of metachromatic secretion granules, in the sensitized guinea pigs challenged with OA (group 2). In the guinea pigs treated with M40403 6 h before OA challenge (group 5), mast cell optical density was markedly higher than in the vehicle-treated (group 2) or the M40404-treated (group 7) animals.

Reduction by M40403 of OA-induced eosinophilic lung infiltration

Detection of MBP-positive eosinophils showed almost no cells in the naïve guinea pigs (group 1). Immunostained cells increased markedly in the sensitized, vehicle-treated, OA-challenged guinea pigs (group 2). They were mostly located in the inflammatory infiltrate surrounding the bronchi and within the stromal septa. In the sensitized, OA-challenged guinea pigs pretreated with M40403 (group 5), but not in those given the inactive enantiomer M40404 (group 7), immunostained eosinophils were markedly reduced, thus attaining values similar to the naïve animals (Fig. 7).

Prevention by M40403 of OA-induced airway epithelial cell apoptosis

Representative micrographs of airway epithelial cells upon TUNEL assay are shown in Fig. 8. No positive cells were detected in the naïve animals (group 1). Conversely, in the sensitized, vehicle-treated guinea pigs (group 2), OA challenge led to the appearance of several airway epithelial

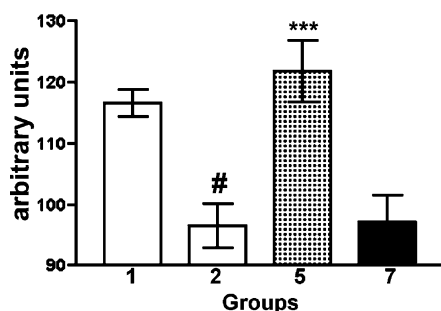


Fig. 6. Optical density of lung mast cells upon OA challenge in naïve guinea pigs (group 1); sensitized, vehicle-treated guinea pigs (group 2); sensitized guinea pigs treated with M40403 (1 mg/kg bw) 6 h before OA challenge (group 5); sensitized guinea pigs treated with M40404 (1 mg/kg bw) 6 h before OA challenge (group 7). [#] $P < 0.001$ vs naïve animals, ^{***} $P < 0.001$ vs vehicle-treated animals.

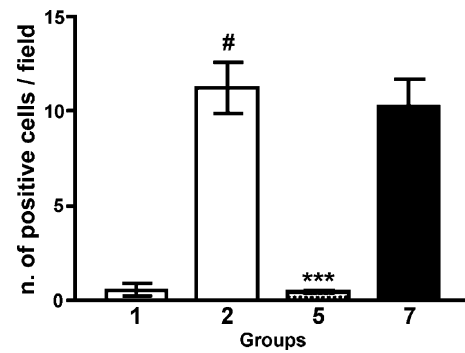


Fig. 7. Number of lung tissue MBP-positive eosinophils upon OA challenge in naïve guinea pigs (group 1); sensitized, vehicle-treated guinea pigs (group 2); sensitized guinea pigs treated with M40403 (1 mg/kg bw) 6 h before OA challenge (group 5); sensitized guinea pigs treated with M40404 (1 mg/kg bw) 6 h before OA challenge (group 7). [#] $P < 0.001$ vs naïve animals, ^{***} $P < 0.001$ vs vehicle-treated animals.

cells undergoing apoptosis. Pretreatment with M40403 6 h before OA aerosol (group 5) resulted in a protection against airway epithelial cell apoptosis.

Reduction by M40403 of OA-induced MPO, MDA, and 8-OHdG elevation in lung tissue

MPO activity (Fig. 9A), a marker of leukocyte infiltration into inflamed tissues, MDA (Fig. 9B), a marker of free radical-mediated inflammatory damage, and 8-OHdG, a

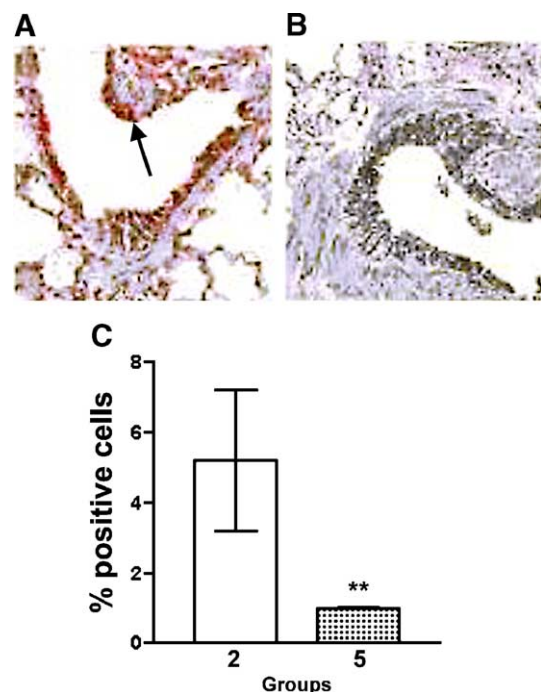


Fig. 8. Number of TUNEL-positive airway epithelial cells (some of them pointed by arrow) upon OA challenge in: (A) sensitized, vehicle-treated guinea pigs (group 2); (B) sensitized guinea pigs treated with M40403 (1 mg/kg bw) 6 h before OA challenge (group 5). M40403 markedly reduces the amount of apoptotic cells. Panel C represents the percentage of TUNEL-positive epithelial cells in the above conditions. ^{**} $P < 0.01$.

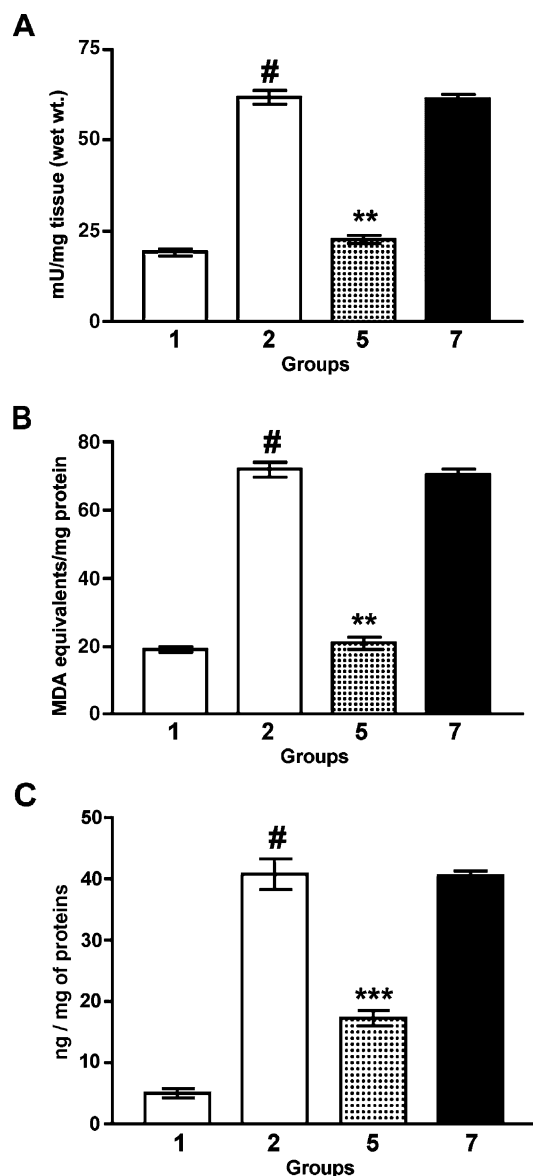


Fig. 9. (A) Myeloperoxidase (MPO), (B) malonyldialdehyde (MDA), and (C) 8-hydroxy-2'-deoxyguanosine (8-OHdG) in lung tissue. These parameters are increased in sensitized, vehicle-treated guinea pigs upon OA challenge (group 2). M40403 (1 mg/kg bw) given 6 h before OA challenge (group 5) evenly reduces MPO, MDA, and 8-OHdG, whereas its inactive enantiomer M40404 (1 mg/kg bw) did not (group 7). # $P < 0.01$ vs naïve animals (group 1); ** $P < 0.01$ and *** $P > 0.001$ vs sensitized, vehicle-treated animals.

marker of free radical induced DNA damage (Fig. 9C), underwent a clear-cut, significant increase in the lung tissue from sensitized, vehicle-treated guinea pigs challenged with OA (group 2) as compared with the naïve controls (group 1). In the sensitized guinea pigs pretreated with M40403 6 h before OA challenge (group 5), MPO, MDA, and 8-OHdG were markedly and significantly decreased compared with the sensitized, vehicle-treated animals (group 2), thus attaining values similar to those of the controls (group 1). On the other hand, in the guinea pigs given M40404 (group 7), MPO, MDA, and 8-OHdG remained high.

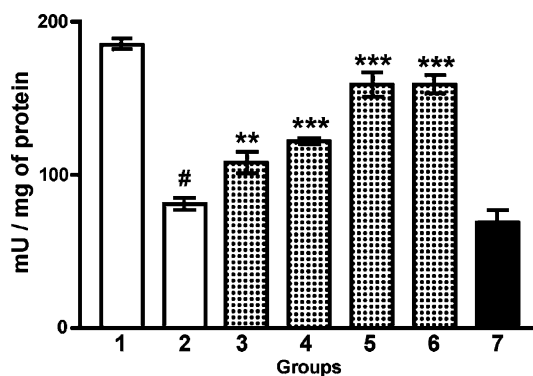


Fig. 10. Effects of M40403 (1 mg/kg bw), given 1, 3, 6, and 9 h before OA challenge (groups 3–6, dotted bars) and of its inactive enantiomer M40404 (1 mg/kg bw), given 6 h before OA challenge (group 7, black bar) on MnSOD activity. The activity of MnSOD, which is strongly decreased in the sensitized, vehicle-treated animals (group 2), is restored by M40403 with a maximum effect at 6 h (group 5). Data are expressed as mean $\pm$ SE of 8 experiments. # $P < 0.001$ vs naïve animals (group 1); ** $P < 0.05$, *** $P < 0.001$ vs sensitized, vehicle-treated animals (group 2).

Reduction by M40403 of OA-induced decrease in MnSOD activity

Challenge with OA of sensitized, vehicle-treated guinea pigs (group 2) caused a marked decrease in lung MnSOD activity as compared with the naïve animals (group 1). Pretreatment with M40403 restored the MnSOD activity with a maximum effect when the drug was given 6 h before OA challenge. M40404 was inactive (Fig. 10).

Reduction by M40403 of PGD₂ in bronchoalveolar lavage

PGD₂, generated by activated mast cells during allergic response, was increased in the bronchoalveolar lavage fluid from sensitized, vehicle-treated guinea pigs challenged with OA (group 2) as compared with the naïve controls (group 1). In the sensitized, OA-challenged guinea pigs given M40403

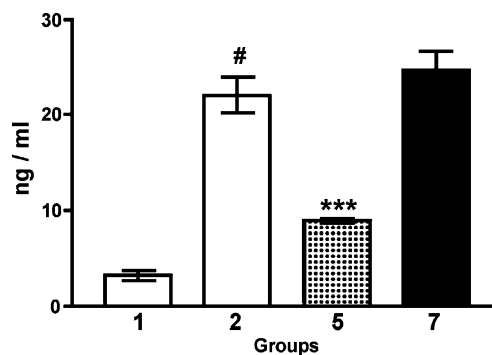


Fig. 11. Levels of mast cell-derived PGD₂ in bronchoalveolar lavage fluid of guinea pigs following OA challenge: naïve guinea pigs (group 1); sensitized, vehicle-treated guinea pigs (group 2); sensitized guinea pigs treated with M40403 (1 mg/kg bw) 6 h before OA challenge (group 5); sensitized guinea pigs treated with M40404 (1 mg/kg bw) 6 h before OA challenge (group 7). # $P < 0.001$ vs naïve animals, *** $P < 0.001$ vs vehicle-treated animals.

(group 5), but not in those given M40404 (group 7), the levels of PGD₂ in the bronchoalveolar fluid were significantly lower than in those of the animals of group 2 (Fig. 11).

Discussion

This study provides evidence that the SOD mimetic compound M40403 is able to counteract the allergen-induced functional, biochemical, and histopathological lung changes in a guinea pig model of allergic asthma-like reaction, similar to that used previously to test substances with antiasthmatic properties [21–24]. Systemic pretreatment of OA-sensitized guinea pigs with M40403 1 to 9 h before OA challenge caused a marked, significant reduction of the occurrence of respiratory abnormalities (cough and dyspnea), bronchoconstriction, alveolar hyperinflation, leukocyte and eosinophilic infiltration, mast cell activation (degranulation and PGD₂ generation), and free radical-induced injury (MDA and 8-OHdG), and epithelial cell apoptosis as compared with the vehicle-treated controls. Moreover, M40403 is able to sustain lung MnSOD activity, which is markedly reduced in the vehicle-treated controls, reaching its peak effect at 6 h from administration. The effect of M40403 appears to depend on its specific SOD mimetic property and the removal of superoxide anion, since the inactive congener M40404 had no protective effect. Thus, the present findings also give indirect support to the involvement of superoxide anion in the pathophysiology of asthma. Our results are in agreement with the recent observation that transgenic mice with elevated levels of Cu/Zn SOD in the lungs are resistant to allergen-induced changes in airway function [20].

It has been reported that inflammatory cells produce large amounts of superoxide anions in asthma and that an inverse correlation exists between superoxide production from leukocytes and FEV₁, suggesting that airway obstruction in asthma is associated with increased production of superoxide anions by leukocytes [39]. In the present study, we have observed that lung leukocyte infiltration, evaluated as MPO activity, and eosinophilic accumulation, evaluated as MBP-positive cells, were significantly reduced in the guinea pigs treated with M40403, thus confirming our previous results obtained with this substance in other models of acute and chronic inflammation [26–28]. As known, infiltration of leukocytes, especially eosinophils, is a prominent feature of asthmatic lungs, and the inflammatory mediators released by these cells give a major contribution to the pathogenesis of allergic asthma [40]. Therefore, the reduction of leukocyte infiltration may be a key mechanism for the beneficial effect of M40403. Besides leukocyte infiltration, pretreatment with M40403 also prevented mast cell activation, since it reduced degranulation and release of PGD₂. In turn, mast cell mediators may cause bronchoconstriction and smooth muscle cell proliferation and may

recruit other inflammatory cells, thereby sparking a vicious circle that may amplify the pathophysiological features of asthma. Since superoxide anions stimulate mast cells to release histamine [41], the present findings suggest that M40403 may hinder mast cell activation by catabolizing superoxide anions, thus having an important preventative effect in the acute airway response to allergens, which is known to depend on eosinophils as well as on mast cells [42]. It is even possible that M40403 may inhibit superoxide-induced activation of high-affinity IgE receptors, as previously reported [43]. While evidence for mast cells as key players in acute allergic reactions is well documented, their role in the late-phase response is controversial. Studies in transgenic mast cell-deficient mice have shown that these cells may not be essential to the asthma-like reaction in mice, but can represent an important local amplifier of antigen-dependent bronchoconstriction [44]. In fact, even in mast cell-deficient mice, sensitization agents can produce strong nonspecific responses, most likely due to the use of artificial adjuvants in many sensitization protocols which promote a strong antibody response and influence the features of this asthma-like model [44]. In human asthmatic lungs, mast cell number is increased and bronchial hyper-responsiveness correlates with the number of these cells. Endobronchial biopsies show increased mast cell degranulation in asthmatic patients compared with nonasthmatic subjects [45–47]. Moreover, the levels of PGD₂, the major cyclooxygenase product generated by activated mast cells during allergic response [38], are elevated in urine of asthmatic patients after antigen exposure in both the early and the late phases, suggesting an involvement of these cells in any phase of the asthmatic response [48].

Taken together, the present findings strongly support SOD mimetics as a valid therapeutic approach for the treatment of asthma, as well as of other bronchopulmonary diseases characterized by the overt production of superoxide anion.

Acknowledgments

This work was supported in part by a grant (PRIN 2002) from the Italian Ministry for Education, University, and Research (MIUR), and by funds from the University of Florence, Italy.

References

- [1] Rennard, S. I. Repair mechanisms in asthma. *J. Allergy Clin. Immunol.* **98**:S278–S286; 1996.
- [2] Bast, A.; Haenen, G. R.; Doelman, C. J. Oxidants and antioxidants: state of the art. *Am. J. Med.* **91**:S2–S13; 1991.
- [3] Calhoun, W. J.; Reed, H. E.; Moest, D. R.; Stevens, C. A. Enhanced superoxide production by alveolar macrophages and air-space cells, airway inflammation, and alveolar macrophage density changes after segmental antigen bronchoprovocation in allergic subjects. *Am. Rev. Respir. Dis.* **145**:317–325; 1992.

- [4] Wu, W.; Samoszuk, M. K.; Comhair, S. A.; Thomassen, M. J.; Farver, C. F.; Dweik, R. A.; Kavuru, M. S.; Erzurum, S. C.; Hazen, S. L. Eosinophils generate brominating oxidants in allergen-induced asthma. *J. Clin. Invest.* **105**:1455–1463; 2000.
- [5] Rahman, I.; Smith, C. A.; Lawson, M. F.; Harrison, D. J.; Macnee, W. Induction of gamma-glutamylcysteine synthetase by cigarette smoke is associated with AP-1 in human alveolar epithelial cells. *FEBS Lett.* **396**:21–25; 1996.
- [6] Comhair, S. A.; Lewis, M. J.; Bhathena, P. R.; Hammel, J. P.; Erzurum, S. C. Increased glutathione and glutathione peroxidase in lungs of individuals with chronic beryllium disease. *Am. J. Respir. Crit. Care Med.* **159**:1824–1829; 1999.
- [7] Comhair, S. A.; Bhathena, P. R.; Dweik, R. A.; Kavuru, M.; Erzurum, S. C. Rapid loss of superoxide dismutase activity during antigen-induced asthmatic response. *Lancet* **355**:624; 2000.
- [8] Page, S.; Ammit, A. J.; Black, J. L.; Armour, C. L. Human mast cell and airway smooth muscle cell interactions: implications for asthma. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **281**:L1313–L1323; 2001.
- [9] Brightling, C. E.; Bradding, P.; Symon, F. A.; Holgate, S. T.; Wardlaw, A. J.; Pavord, I. D. Mast-cell infiltration of airway smooth muscle in asthma. *N. Engl. J. Med.* **346**:1699–1705; 2002.
- [10] Rossi, G. L.; Olivieri, D. Does the mast cell still have a key role in asthma? *Chest* **112**:523–529; 1997.
- [11] Bowler, R. P.; Crapo, J. D. Oxidative stress in airways. Is there a role for extracellular superoxide dismutase? *Am. J. Respir. Crit. Care Med.* **166**:S38–S43; 2002.
- [12] Bowler, R. P.; Crapo, J. D. Oxidative stress in allergic respiratory diseases. *J. Allergy Clin. Immunol.* **110**:349–356; 2002.
- [13] Barnes, P. J. Reactive oxygen species and airway inflammation. *Free Radic. Biol. Med.* **9**:235–243; 1990.
- [14] Smith, L. J.; Shamsuddin, M.; Sporn, P. H.; Denenberg, M.; Anderson, J. Reduced superoxide dismutase in lung cells of patients with asthma. *Free Radic. Biol. Med.* **22**:1301–1307; 1997.
- [15] Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. Apparent hydroxyl radical production by peroxynitrite: implication for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87**:1620–1624; 1990.
- [16] Yamakura, F.; Taka, H.; Fujimura, T.; Murayama, K. Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J. Biol. Chem.* **273**:14085–14089; 1998.
- [17] Macmillan-Crow, L. A.; Thompson, J. A. Tyrosine modifications and inactivation of active site manganese superoxide dismutase mutant (y34f) by peroxynitrite. *Arch. Biochem. Biophys.* **66**:82–88; 1999.
- [18] Macmillan-Crow, L. A.; Cruthirds, D. L. Invited review: manganese superoxide dismutase in disease. *Free Radic. Res.* **34**:325–336; 2001.
- [19] Yamakura, F.; Matsumoto, T.; Fujimura, T.; Taka, H.; Murayama, K.; Imai, T.; Uchida, K. Modification of a single tryptophan residue in human Cu,Zn-superoxide dismutase by peroxynitrite in the presence of bicarbonate. *Biochim. Biophys. Acta* **1548**:38–46; 2001.
- [20] Larsen, G. L.; White, C. W.; Takeda, K.; Loader, J. E.; Nguyen, D. D.; Joetham, A.; Groner, Y.; Gelfand, E. W. Mice that overexpress Cu/Zn superoxide dismutase are resistant to allergen-induced changes in airway control. *Am. J. Physiol. (Lung Cell Mol. Physiol.)* **279**:L350–L359; 2000.
- [21] Bani, D.; Ballati, L.; Masini, E.; Bigazzi, M.; Bani Sacchi, T. Relaxin counteracts asthma-like reaction induced by inhaled antigen in sensitised guinea pigs. *Endocrinology* **138**:1909–1915; 1997.
- [22] Xiang, A.; Uchida, Y.; Nomura, A.; Ijima, H.; Dong, F.; Zhang, M. J.; Hasegawa, S. Effects of airway inflammation on cough response in the guinea pig. *J. Appl. Physiol.* **85**:1847–1854; 1998.
- [23] Liu, Q.; Fujimura, M.; Tachibana, H.; Myou, S.; Kasahara, K.; Yasui, M. Characterization of increased cough sensitivity after antigen challenge in guinea pigs. *Clin. Exp. Allergy* **31**:474–484; 2001.
- [24] Suzuki, Y.; Masini, E.; Mazzocca, C.; Cuzzocrea, S.; Ciampa, A.; Suzuki, H.; Bani, D. Inhibition of poly(ADP-ribose) polymerase prevents allergen-induced asthma-like reaction in sensitised guinea pigs. *J. Pharmacol. Exp. Ther.* **311**:1241–1248; 2004.
- [25] Riley, D. P.; Lennon, P. J.; Neumann, W. L.; Weiss, R. H. Toward the rational design of superoxide dismutase mimics: mechanistic studies for the elucidation of substituent effects on the catalysis activity of macrocyclic manganese (II) complexes. *J. Am. Chem. Soc.* **119**:6522–6528; 1997.
- [26] Salvemini, D.; Wang, Z. Q.; Zweier, J. L.; Samouilov, A.; Macarthur, H.; Misko, T. P.; Currie, M. G.; Cuzzocrea, S.; Sikorski, J. A.; Riley, D. P. A non-peptidyl mimic of superoxide dismutase with therapeutic activity in rats. *Science* **286**:304–306; 1999.
- [27] Cuzzocrea, S.; Mazzon, E.; Dugo, L.; Caputi, A. P.; Riley, D. P.; Salvemini, D. Protective effects of M40403, a superoxide dismutase mimetic, in a rodent model of colitis. *Eur. J. Pharmacol.* **432**:79–89; 2001.
- [28] Masini, E.; Cuzzocrea, S.; Mazzon, E.; Mazzocca, C.; Mannaioni, P. F.; Salvemini, D. Protective effects of M40403, a selective superoxide dismutase mimetic, in myocardial ischaemia and reperfusion injury in vivo. *Br. J. Pharmacol.* **136**:905–917; 2002.
- [29] Ballati, L.; Evangelista, S.; Manzini, S. Repeated antigen challenge induced airway hyperresponsiveness to neurokinin A and vagal non-adrenergic, non-cholinergic (NANC) stimulation in guinea pigs. *Life Sci.* **51**:L119–L124; 1992.
- [30] Jones, T.; Masson, P.; Hamel, R.; Brunet, G.; Holme, G.; Girard, Y.; Larue, M.; Rokach, J. Biological activity of leukotriene sulfones on respiratory tissues. *Prostaglandins* **24**:279–291; 1982.
- [31] Masini, E.; Bani, D.; Bigazzi, M.; Mannaioni, P. F.; Bani-Sacchi, T. Effects of relaxin on mast cells. In vitro and in vivo studies in rats and guinea pigs. *J. Clin. Invest.* **94**:1974–1980; 1994.
- [32] Bucchieri, F.; Puddicombe, S. M.; Lordan, J. L.; Richter, A.; Buchanan, D.; Wilson, S. J.; Ward, J.; Zummo, G.; Howarth, P. H.; Djukanovic, R.; Holgate, S. T.; Davies, D. E. Asthmatic bronchial epithelium is more susceptible to oxidant-induced apoptosis. *Am. J. Respir. Cell. Mol. Biol.* **27**:179–185; 2002.
- [33] Mullane, K. M.; Kraemer, R.; Smith, B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J. Pharmacol. Methods* **14**:157–167; 1985.
- [34] Arouma, O. I.; Halliwell, B.; Loughton, M. J.; Quinlan, G. J.; Gutteridge, J. M. C. The mechanism of initiation of lipid peroxidation: evidence against a requirement for an iron (II)-iron (III) complex. *Biochem. J.* **258**:617–620; 1989.
- [35] Lodovici, M.; Casalini, C.; Carieggi, R.; Michelacci, L.; Dolara, P. Levels of 8-hydroxydeoxyguanosine as a marker of DNA damage in human leukocytes. *Free Radic. Biol. Med.* **28**:13–17; 2000.
- [36] Beuchamp, C.; Friedovich, I. Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal. Biochem.* **44**:276–287; 1971.
- [37] Nishida, S.; Teramoto, K.; Kimoto-Kinoshita, S.; Tohda, Y.; Nakajima, S.; Tomura, T. T.; Irimajiri, K. Changes of Cu,Zn-superoxide dismutase activity of guinea pig lung in experimental asthma. *Free Radic. Res.* **36**:601–606; 2002.
- [38] Bochenek, G.; Nizankowska, E.; Gielicz, A.; Swierczynska, M.; Szczeklik, A. Plasma 9alpha,11beta-PGF2, a PGD2 metabolite, as a sensitive marker of mast cell activation by allergen in bronchial asthma. *Thorax* **59**:459–464; 2004.
- [39] Jarjour, N. N.; Calhoun, W. J. Enhanced production of oxygen radicals in asthma. *J. Lab. Clin. Med.* **123**:131–136; 1994.
- [40] Kroegel, C.; Liu, M. C.; Hubbard, W. C.; Lichtenstein, L. M.; Bochner, B. S. Blood and bronchoalveolar eosinophils in allergic subjects after segmental antigen challenge: surface phenotype, density heterogeneity, and prostanoic acid production. *J. Allergy Clin. Immunol.* **93**:725–734; 1994.
- [41] Mannaioni, P. F.; Masini, E. The release of histamine by free radicals. *Free Radic. Biol. Med.* **5**:177–197; 1988.

- [42] Wardlaw, A. J.; Dunnette, S.; Gleich, G. J.; Collins, J. V.; Kay, A. Eosinophils and mast cells in bronchoalveolar lavage in subjects with mild asthma. Relationship to bronchial hyperreactivity. *Am. Rev. Respir. Dis.* **137**:62–69; 1988.
- [43] Yoshimaru, T.; Suzuki, Y.; Matsui, T.; Yamashita, K.; Ochiai, T.; Yamachi, M.; Shimizu, K. Blockade of superoxide generation prevents high-affinity immunoglobulin E receptor-mediated release of allergic mediators by rat mast cell line and human basophils. *Clin. Exp. Allergy* **32**:612–618; 2002.
- [44] Williams, C. M.; Galli, S. J. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J. Exp. Med.* **192**:455–462; 2000.
- [45] Beasley, R.; Roche, W. R.; Roberts, J. A.; Holgate, S. T. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am. Rev. Respir. Dis.* **139**:806–817; 1989.
- [46] Djukanovic, R.; Wilson, J. W.; Britten, K. M.; Wilson, S. J.; Walls, A. F.; Roche, W. R.; Howarth, P. H.; Holgate, S. T. Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am. Rev. Respir. Dis.* **145**:669–674; 1992.
- [47] Pesci, A.; Foresi, A.; Bertorelli, G.; Chetta, A.; Olivieri, D.; Oliveri, D. Histochemical characteristics and degranulation of mast cells in epithelium and lamina propria of bronchial biopsies from asthmatic and normal subjects. *Am. Rev. Respir. Dis.* **147**:684–689; 1993.
- [48] O'Sullivan, S. On the role of PGD₂ metabolites as markers of mast cell activation in asthma. *Acta Physiol. Scand. Suppl.* **644**:1–74; 1999.