



## Original Contribution

Suppression of allergen-induced respiratory dysfunction and airway inflammation in sensitized guinea pigs by  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ , a novel superoxide scavenger compoundLorenzo Cinci<sup>a,b</sup>, Emanuela Masini<sup>b</sup>, Andrea Bencini<sup>c</sup>, Barbara Valtancoli<sup>c</sup>, Rosanna Mastroianni<sup>b</sup>, Laura Calosi<sup>a</sup>, Daniele Bani<sup>a,\*</sup><sup>a</sup> Department of Anatomy, Histology and Forensic Medicine, Section of Histology Italy<sup>b</sup> Department of Preclinical and Clinical Pharmacology Italy<sup>c</sup> Department of Chemistry; University of Florence, Florence, Italy

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## ABSTRACT

Reactive oxygen species produced during allergic inflammation are key players of the pathophysiology of asthma, leading to oxidative tissue injury and inactivation of endogenous manganese superoxide dismutase (MnSOD). On this ground, removal of excess superoxide anion by scavenger molecules would be beneficial and protective. Here we show that a novel manganese(II)-containing polyamine-polycarboxylic compound, termed  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ , with potent superoxide dismuting properties decreases the respiratory and histopathological lung abnormalities due to allergen inhalation in a model of ovalbumin (OA)-induced allergic asthma-like reaction in sensitized guinea pigs.

Severe respiratory dysfunction in response to OA aerosolic challenge arose rapidly in the sensitized animals and was accompanied by bronchoconstriction, alveolar hyperinflation, mast cell activation, increased leukocyte infiltration (evaluated by myeloperoxidase assay), oxidative lung tissue injury (evaluated by the thiobarbituric-acid-reactive substances and nitrotyrosine immunostaining), decay of endogenous MnSOD activity, production of pro-inflammatory prostaglandins, and lung cell apoptosis. Treatment with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (15 mg/kg, given 1 h before allergen challenge), but not the inactive congener  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  lacking redox-active metal site, significantly attenuated all the above functional, histopathological and biochemical parameters of allergic inflammation and restored the levels of MnSOD activity. In conclusion, our findings support the potential therapeutic use of  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  as novel superoxide scavenger drug in asthma and anaphylactic reactions.

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## Introduction

Bronchial hyperresponsiveness and inflammation are the hallmarks of acute asthma and triggering events of adverse airway remodelling, which includes loss of airway epithelium, goblet cell and

smooth muscle cell hyperplasia, and abnormal collagen synthesis [1]. A large body of evidence indicates that increased levels of reactive oxygen species (ROS) produced by inflammatory and epithelial cells in the airways, including chiefly superoxide anion ( $\text{O}_2^{\cdot-}$ ) and hydroxyl radical, are key players in the pathogenesis of asthma [2–4]. In animal models, ROS can closely reproduce the dysfunctional and histopathological features of asthma [2]. In asthmatic patients, the degree of bronchial obstruction is paralleled by increased ROS generation in the airways lumen [3]. On the other hand, the levels of lung endogenous antioxidants, including manganese superoxide dismutase (MnSOD) [5–7], are rapidly inactivated in the airways of asthmatic subjects during the transition from acute to chronic disease, thus rendering the asthmatic lung prone to oxidative injury [8–11]. Moreover,  $\text{O}_2^{\cdot-}$  can challenge mast cells to release the pro-inflammatory mediators, including histamine, prostaglandin  $\text{D}_2$ , cysteinyl leukotrienes and tryptase [12–14], thereby exaggerating the lung inflammatory response [15]. Finally,  $\text{O}_2^{\cdot-}$  and nitric oxide, which are both massively generated during inflammation, combine to form peroxynitrite (PN), a strong oxidizing and nitrating agent and crucial player of lung tissue injury [5].

**Abbreviations:** BAL, bronchoalveolar lavage; 6-ketoPG1 $\alpha$ , 6-keto-prostaglandin 1 $\alpha$ ; LTB $_4$ , leukotriene B $_4$ ; M 40403, [manganese(II)dichloro{(4R,9R,14R,19R)-3,10,13,20,26-pentaazatetracyclo[20.3.1.0.<sup>4,9</sup>0.<sup>14,19</sup>]hexacos-1(26),-22(23),24-triene)}]; salen, salicylaldehyde-ethylene diamine Schiff base; EUK 134, manganese(III) [metoxy-N,N'-bis(salicyldene)ethylenediamine chloride]; MnTBAP $^{3-}$ , manganese(III) [meso-tetrakis(p-carboxylatophenyl)porphyrin] (AEOL-10201); MnTE-2-PyP $^{5+}$ , manganese(III) [meso-tetrakis(N-ethylpyridinium-2-yl)porphyrin] (AEOL10113);  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ , manganese(II) 4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetate; HEDTA $^{3-}$ , N-(2-hydroxyethyl)ethylenediamine-N,N'-triacetate; EGTA $^{4-}$ , ethylene-bisglycol-tetraacetate; MnSOD, manganese superoxide dismutase; OA, ovalbumin; PAO, pressure at alveolar opening; PGD $_2$ , prostaglandin D $_2$ ; ROS, reactive oxygen species; TBARS, thiobarbituric acid-reactive substances;  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ , zinc(II) 4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetate.

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In this context, it is conceivable that any pharmacological intervention aimed at reducing the excess ROS in asthma, including administration of exogenous recombinant MnSOD [16] as well as synthetic SOD-mimetic compounds [17,18], could have beneficial effects. In principle, extractive or recombinant SOD appeared as the most logical choice for such a causative therapeutic approach [19]. However, its actual clinical use has been hampered by multiple factors, including solution instability, limited cellular accessibility, immunogenicity, short half-life, and high production costs [16,20]. Because of these limitations, pharmacological research pointed at metallic ion chelates with low molecular weight organic scaffolds capable of catalyzing dismutation of  $O_2^-$  like authentic SOD [17].

In this view, several manganese complexes with ligands containing nitrogen donors have been developed as potential anti-inflammatory agents for different pathologies, including lung diseases [6]. The most representative ones are sketched in Fig. 1. Among these molecules,  $Mn^{II}$  complexes with pentaazamacrocyclic ligands capable to selectively dismutate  $O_2^-$  [21,22], such as M40403, have been proven effective to reduce the allergic asthma-like reaction to inhaled allergen in sensitized guinea pigs [18]. Similarly,  $Mn^{III}$  complexes with synthetic *meso*-substituted porphyrins, such as AEOL-10201, are capable to scavenge  $O_2^-$ , as well as  $H_2O_2$ , PN, and lipid peroxides [6,23], depending on the chemical substituents at the porphyrin ring [24]. Moreover,  $Mn^{III}$  complexes with salen (salicylaldehyde-ethylene diamine Schiff base) derivatives, such as EUK-134, have been also successfully used as anti-oxidant agents, as they have both  $O_2^-$  and  $H_2O_2$  dismutation activities [6,25]. Like the metalloporphyrins, these compounds are not selective and can also react with PN [26].

In the last twenty years, the above classes of manganese complexes have been extensively studied as anti-oxidants and therapeutics. Less attention has been paid to  $Mn^{II}$  complexes with polyamine-polycarboxylate ligands, despite the notion that, in principle, polyamine-polycarboxylate scaffolds may represent an optimal tool for the

synthesis of stable  $Mn^{II}$  complexes. In fact, these compounds can form highly stable metal chelates with  $Mn^{II}$  [27], are resistant to oxidizing and reducing agents, are highly soluble in aqueous media, and have low *in vitro* and *in vivo* toxicity, all properties that render them suitable for several biological and medicinal applications [28–30].

As a first approach to the study of polyamine-polycarboxylate  $Mn^{II}$  complexes as potential ROS-scavenger therapeutics, we focused on 1,4,7,10-tetraazacyclododecane-1,7-diacetic acid ( $H_2DO_2A$ ) and 4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetic acid ( $H_2Me_2DO_2A$ ), the latter synthesized in our laboratories [31]. In aqueous solution, their carboxylic groups deprotonates upon  $Mn^{II}$  binding, yielding  $Mn^{II}(DO_2A)$  and  $Mn^{II}(Me_2DO_2A)$  neutral complexes [31]. Moreover, binding of the carboxylate groups to  $Mn^{II}$  markedly increases stability of the complex with respect to simple polyamine ligands [32]. The stability of  $Mn^{II}(DO_2A)$  and  $Mn^{II}(Me_2DO_2A)$  is also higher than that of the complexes of manganese with biological ligands present in the cellular environment, such as ATP and glutathione [33,34], thus largely preventing the dechelation of  $Mn^{II}(DO_2A)$  and  $Mn^{II}(Me_2DO_2A)$  with biological chelators. In our previous study, we demonstrated that only  $Mn^{II}(Me_2DO_2A)$  had strong  $O_2^-$ -scavenging properties *in vitro*, whereas both  $Mn^{II}(DO_2A)$  and the  $Mn^{II}$  complexes with charged complexes (N-(2-hydroxyethyl)ethylenediamine-N,N',N'-tri-acetic acid or ethylene-bisglycol-tetracetic acid) were poorly or not effective [31]. We also showed that the presence of two anionic carboxylate groups neutralizing the charge of  $Mn^{II}$  and two lipophilic methyl groups render  $Mn^{II}(Me_2DO_2A)$  capable to cross the cell membranes and attain significant intracellular concentrations [31], thus being capable to directly scavenge  $O_2^-$  in the cytoplasmic environment. In this context, the cyclic tetraamine structure of  $Mn^{II}(Me_2DO_2A)$  may offer a further advantage, as it is easily accessible to N-functionalization of the secondary amine groups in 4,10 position, for instance to increase the lipophilic characteristics of the compounds by substituting hydrophobic alkyl or aryl moieties to the methyls [35].

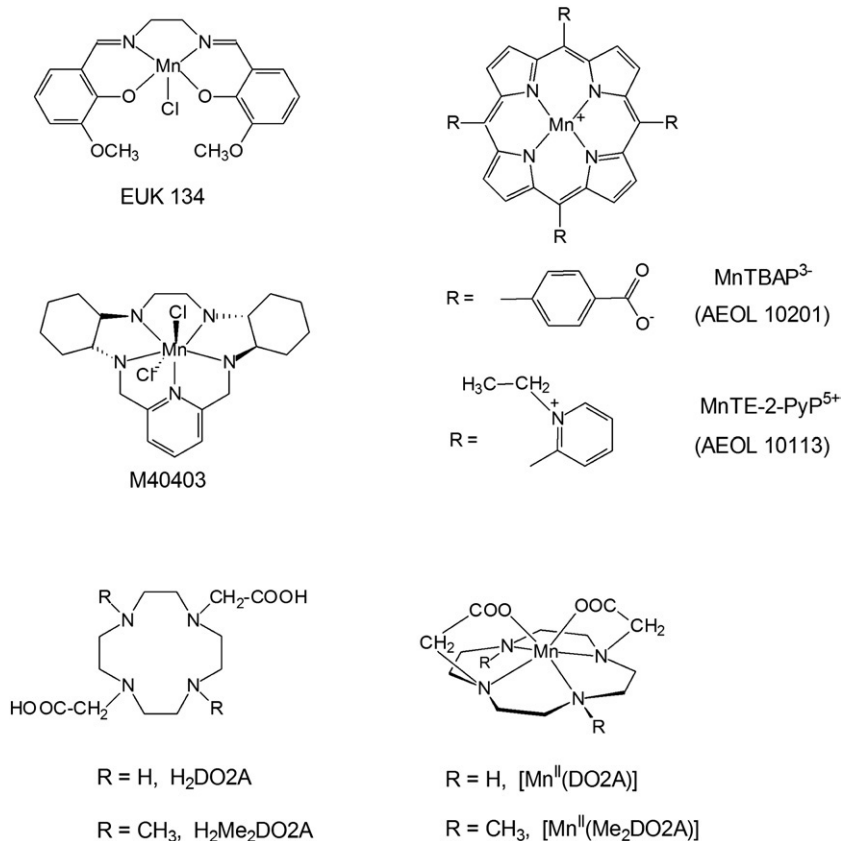


Fig. 1. Structure of the manganese complexes with ligands containing nitrogen donors, including the  $Mn^{II}(Me_2DO_2A)$  used in this study.

The current study was designed to test the therapeutic potential of  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ , using a well-established *in vivo* model of allergic asthma-like reaction in ovalbumin(OA)-sensitized guinea pigs [18,36–38]. This compound should offer substantial theoretical advantages over MnSOD because it does not require enzyme specificity and meets some major pharmacological efficiency criteria: i) effectiveness in the nanomolar/micromolar range in *in vitro* cellular models, ii) activity and stability in biological systems, iii) lack of toxicity [31]. As control, we used the inactive congener  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ , in which the functional  $\text{Mn}^{\text{II}}$  was replaced with a  $\text{Zn}^{\text{II}}$  ion with no capability to catalyze decomposition of  $\text{O}_2^-$ .

## Materials and methods

### Reagents

The chemicals used were obtained from Sigma-Aldrich (Milan, Italy), unless otherwise specified. Ovalbumin (OA) was from Fluka (Buchs, Switzerland). The  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  compound,  $\text{Mn}^{\text{II}}$  4,10-dimethyl-1,4,7,10-tetraazacyclododecane-1,7-diacetate (Fig. 1), was synthesised in the laboratories of the Department of Chemistry, University of Florence, Italy, and is currently pending for Italian and international patent [35]. The inactive congener  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ , in which functional  $\text{Mn}^{\text{II}}$  was replaced with inactive  $\text{Zn}^{\text{II}}$ , was synthesised by a similar procedure.

### Animals

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

**Group 1** ( $n=9$ ): the animals were injected with isotonic saline (5 ml/kg i.p., plus 5 ml/kg s.c.) and 18–21 days later, 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 i.p. plus 100 mg/kg, s.c. 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 check sensitization by the occurrence of allergen-elicited breathing abnormalities and then quickly withdrawn from antigen exposure. The guinea pigs reactive to the inhaled antigen were evenly divided among the following experimental groups:

**Group 2** ( $n=9$ ). No further treatment. These animals are referred to as sensitized unchallenged and were also used as negative controls.

**Group 3** ( $n=9$ ). Treatment with an i.p. injection of 0.5 ml of vehicle (phosphate-buffered saline, PBS, pH 7.4) 1 hour before undergoing OA provocation test, as described below. They are referred to as sensitized, OA challenged animals.

**Group 4** ( $n=9$ ). Treatment with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  at the dose of 15 mg/kg, dissolved in 0.5 ml PBS and injected i.p. 1 hour before OA provocation test.

**Group 5** ( $n=9$ ): Treatment with  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  at the dose of 15 mg/kg, dissolved in 0.5 ml PBS and injected i.p. 1 hour before OA provocation test.

In previous experiments carried out in mouse and rat models of acute and chronic inflammation [24], we assayed multiple  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  doses (1, 5 and 15 mg/kg). These studies have indicated the 15 mg/kg dose as having marked anti-inflammatory properties and no toxic effects; therefore, this dose was chosen for the present study.

### Evaluation of respiratory activity

In a first experimental subset, 6 guinea pigs from each group were individually placed in an airtight whole-body plethysmographic chamber, as previously described [18,36–38]. The breathing-induced changes in inner chamber pressure were registered with a high sensitivity pressure transducer (P75 type 379, HSE, Germany) connected to a polygraph (Universal oscillograph, Harvard, UK). Upon breath stabilization, usually occurring within 30–60 seconds, guinea pigs were challenged with the OA aerosol for 10 sec. 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. Breathing of the animals subjected to the different treatments was registered for 10 min. after the onset of aerosol administration and respiratory abnormalities were quantified according to the previously reported criteria [18,36–38]. The following parameters were evaluated: i) latency time (sec.) 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 (sec.) 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 lung tissue samples from the middle and the lower lobes were taken for biochemical and morphological analyses, as described below.

### Measurement of bronchoconstriction

In a second experimental subset, the remaining 3 guinea pigs from each group were used to assess bronchoconstriction by a mechanical ventilation method, as described [39]. The animals were given sodium penthotal (100 mg/kg, Abbott, Latina, Italy) to induce anaesthesia and abolish natural respiration. Body temperature was monitored continuously and maintained constant at 34.5 °C. The trachea was cannulated with a polyethylene tube and the animals 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. Changes in lung resistance to inflation (pressure at the airway opening; PAO), specifically the lateral pressure of the inlet air tube which is directly related to airway resistance, were registered by the same pressure transducer/polygraph apparatus described above. After 10 min. 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 were recorded for 10 min after the beginning of OA aerosol and expressed as percent changes over the basal values. These guinea pigs were also used to perform bronchoalveolar lavage (BAL). To this end, a cannula was inserted into the trachea and 3 ml of PBS, pH 7.4, were injected and flushed 3 times before collection of BAL fluid. This was then centrifuged at 1100 g for 30 min., the cell-free supernatant collected, its volume measured and frozen at –70 °C until needed for biochemical assays, as reported below.

### Histological and morphometrical analysis

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  $\mu\text{m}$  thick, were stained with hematoxylin and eosin and used 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 analysed. At the chosen magnification, each field corresponds to a tissue area of  $570,224 \mu\text{m}^2$  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 (test area:  $125,400 \mu\text{m}^2$ ). For both alveolar and bronchial luminal areas, digital micrographs of the microscopical fields to be analysed were taken and surface area measurements were carried out using the ImageJ 1.33 free-share image analysis software (<http://rsb.info.nih.gov/ij>) upon appropriate threshold to include only blank, tissue-free air spaces. The mean values ( $\pm$  SEM) of alveolar and bronchial luminal areas were then calculated for each experimental group.

### Evaluation of mast cell granule release

This assay was carried out on histological lung tissue sections, 5  $\mu\text{m}$  thick, stained with Astra blue (Fluka, Buchs, Switzerland). This cationic dye binds specifically to heparin contained in mast cell granules and can be used as a probe to evaluate the cells' content of secretion granules by optical density measurements. Digital images of individual mast cells, randomly taken from the 2 different lung samples using a  $\times 100$  oil immersion objective, were analysed with the ImageJ 1.33 software. In each animal, 30 different mast cells were analysed and the mean optical density value ( $\pm$  SEM) was then calculated for the entire experimental group.

### Determination of myeloperoxidase (MPO) activity

This tissue indicator of leukocyte accumulation was determined as previously described [40]. 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  $20,000 g$  at  $4^\circ\text{C}$ . An aliquot of the supernatant was then allowed to react with a solution of tetramethyl-benzidine (1.6 mM) and 0.1 mM  $\text{H}_2\text{O}_2$ . The rate of change in absorbance was measured spectrophotometrically at 650 nm. Myeloperoxidase activity was defined as the quantity of enzyme degrading 1  $\mu\text{mol}$  of peroxide per min at  $37^\circ\text{C}$  and was expressed in mU/mg wet tissue.

### Determination of thiobarbituric acid-reactive substances (TBARS)

TBARS, such as malondialdehyde, are end-products of cell membrane lipid peroxidation by ROS and are considered reliable markers of oxidative tissue injury. They were determined by measurement of the chromogen obtained from the reaction of TBARS with 2-thiobarbituric acid [41]. 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  $2,000 \times g$  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 nmol of TBARS/mg of protein, using a standard curve of 1,1,3,3-tetramethoxypropane.

### Evaluation of nitrotyrosine

Protein tyrosine residue nitration, a marker for PN-mediated damage, was determined by nitrotyrosine (NT) immunostaining on histological lung tissue sections, as described [42]. Sections of formalin-fixed, paraffin-embedded lung specimens were treated with 0.1% trypsin for 10 min. to retrieve antigen, then with 0.3%  $\text{H}_2\text{O}_2$  (v/v) in 60% methanol (v/v) to quench endogenous peroxidase and then incubated overnight with rabbit polyclonal anti-nitrotyrosine antiserum (Upstate Biotechnology, Buckingham, UK; 1:100) at  $4^\circ\text{C}$ . Immune reaction was revealed by goat anti-rabbit IgG conjugated with biotin (Vector Lab, Burlingame, CA, USA; 1:200) followed by incubation with ABC complex (Vector Lab; 1:200). Negative controls were carried out by omitting the primary or the secondary antibodies. Staining was performed in a single session, to minimize artifactual differences in the staining. Photomicrographs of lung tissue were randomly taken using the same digital photomicroscopy apparatus described above with a  $\times 40$  objective. Each microscopic field corresponds to a test area of  $38,700 \mu\text{m}^2$ . On the digitized images (5 per animal), measurements of the surface area and optical density of NT-immunostained tissue was carried out using the ImageJ 1.33 image analysis program. The values, in arbitrary units, have been calculated by the formula; surface area  $\times$  optical density  $\times 10^{-6}$ .

### 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  $100 \times g$  for 10 min. Supernatants were used for SOD measurement. The assay of MnSOD activity was carried out based on SOD-induced inhibition of the conversion of nitro blue tetrazolium (NBT) into formazan mediated by  $\text{O}_2^-$  generated by xanthine oxidase [43]. The reaction was performed in sodium carbonate buffer, 50 mM, pH 10.1, containing 0.1 mM EDTA, 25  $\mu\text{M}$  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 to formazan 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 mU/mg of proteins.

### Determination of $\text{PGD}_2$ , 6-keto $\text{PG}1\alpha$ and $\text{LTB}_4$ in BAL Fluid

Production of  $\text{PGD}_2$ , the major cyclooxygenase product generated by activated mast cells during allergic response, and of 6-keto $\text{PG}1\alpha$  and  $\text{LTB}_4$ , classical inflammatory prostanoids released by activated leukocytes, were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kits (Cayman Chemical, Ann Arbor, MI), following the protocol provided by the manufacturer. Results are expressed as ng substance/ml BAL fluid.

### Determination of caspase-3 activity

The enzymatic activity of caspase-3 was determined using the Ac-Asp-Glu-Val-Asp-AMC (Ac-DEVD-AMC; Bachem) fluorescent substrate [44]. Lung tissue samples were homogenized with 10 mM N-2-

hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes, pH 7.4), containing 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS), 42 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 µg/ml leupeptin, and 1 µg/ml pepstatin A. The homogenate was then centrifuged at 10,000 g for 10 min. The supernatants (containing 250 µg total protein) were incubated with 40 µM of AC-DEVD-AMC for 60 min. at 37 °C. Substrate cleavage was monitored fluorometrically (Spectrofluor JY3 D, Jobin Yvon, Paris, France) at 380 nm excitation and 460 nm emission wave lengths. Data are expressed as arbitrary units/mg proteins. One unit of enzyme activity is defined as the amount of enzyme required to liberate 40 µmol of Ac-DEVD-AMC upon 60 min. at 37 °C.

#### Statistical analysis

The reported data are expressed as the mean ± SEM of independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Student-Newman-Keuls multiple comparison *post hoc* test, *p* < 0.05 was considered significant. Calculations were done using a GraphPad Prism 4.0 statistical program (GraphPad software, San Diego, CA).

#### Results

##### Suppression by Mn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) of OA-induced respiratory dysfunction and bronchoconstriction

The mean values of the respiratory parameters assayed are reported in Fig. 2. Breathing recordings from the naïve (group 1) and the sensitized unchallenged (group 2) guinea pigs after inhalation of OA and saline, respectively, showed normal features, apart for sporadic cough strokes due to mechanical stimulation of the airways by the aerosol droplets. On the other hand, challenge of sensitized, vehicle-treated guinea pigs with OA aerosol (group 3) resulted in striking abnormalities in the respiratory pattern, consisting of a significant decrease 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, pretreatment of the sensitized guinea pigs with Mn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) 1 h before OA aerosol (group 4), resulted in a marked, statistically significant attenuation of the degree of respiratory abnormalities as compared with the sensitized, OA challenged animals pretreated with the vehicle alone (group 3), or with those which received the inactive compound Zn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) in the place of the superoxide scavenger (group 5). In particular, pretreatment with Mn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) increased cough latency (Fig. 2A), and decreased cough severity (Fig. 2B), while clear-cut episodes of dyspnea were either absent or markedly delayed as compared with the vehicle-treated animals (group 3) (Fig. 2C). In keeping with these findings, the experiments with mechanically ventilated animals showed that, in the sensitized, vehicle-treated guinea pigs (group 3), OA challenge induced a prompt, marked increase in inflation pressure (PAO) as compared with the negative control groups (1 & 2), indicating the occurrence of bronchoconstriction. This adverse effect of OA was significantly decreased in the animals treated with Mn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) 1 h before the aerosol challenge (group 4), but not in those treated for the same time with the inactive compound Zn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) (group 5) (Fig. 2D).

##### Suppression by Mn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) of OA-induced lung morphological abnormalities

At autopsy, the lungs from the sensitized, vehicle-treated guinea pigs challenged with OA (group 3) appeared swollen and did not deflate upon sectioning of the trachea or of main bronchi, indicating obstruction of peripheral bronchi and entrapment of air in the peripheral alveolar spaces. Similar features were also observed in the

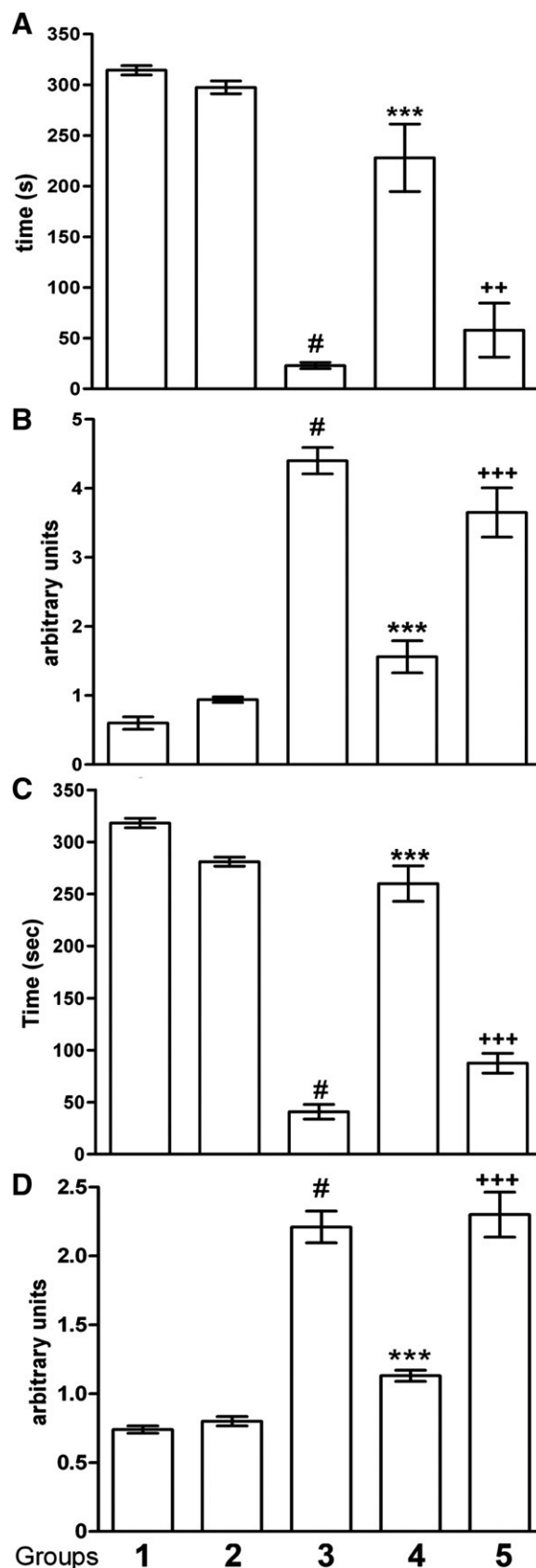


**Fig. 2.** Effects of Mn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) (group 4) and its inactive congener Zn<sup>II</sup>(Me<sub>2</sub>DO<sub>2</sub>A) (group 5), given 1 h before OA challenge, on cough latency (A), cough severity (B), latency for the onset of dyspnea (C) and bronchoconstriction (D) in comparison with the negative controls (groups 1, 2) and the sensitized, vehicle-pretreated animals (group 3). Significance of differences (one-way ANOVA, A-C, *n* = 6; D, *n* = 3): # *p* < 0.001 vs. groups 1 & 2; \*\*\* *p* < 0.001 vs. group 3; ++ *p* < 0.01, +++ *p* < 0.001 vs. group 4.

guinea pigs pretreated with inactive  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 5). Conversely, lung inflation was not observed in the negative controls (groups 1,2) or in the animals pretreated with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  before OA aerosol (group 3).

Histological analysis showed that the lung tissue of the naïve and the sensitized unchallenged guinea pigs (groups 1,2) had a normal appearance. In particular, intrapulmonary bronchioli showed open lumina, with short folds of the mucosa, and alveolar air spaces were evenly small sized (Fig. 3A). Conversely, in the sensitized, OA challenged guinea pigs pretreated with vehicle (group 3) there was a striking reduction of the lumen of intrapulmonary bronchi and a marked dilation of the alveolar air spaces in large areas of the lung parenchyma (Fig. 3B). Similar features were also observed in the animals pretreated with the inactive  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (Fig. 3C). In the guinea pigs pretreated with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 4), the above lung histopathological changes were substantially decreased (Fig. 3D). 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  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  prevents OA-induced bronchiolar constriction and alveolar air space dilatation (Figs. 3E and F).

#### Suppression by $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ of OA-induced mast cell granule release

Compared with the negative controls (groups 1,2; Fig. 4A), a significant decrease in mast cell optical density upon staining with Astra blue was detected in the sensitized, OA challenged guinea pigs pretreated with the vehicle (group 3), indicating secretion granule discharge (Fig. 4B). In the guinea pigs pretreated with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 4; Fig. 4C) mast cell optical density was markedly higher than in the vehicle-pretreated (group 2) or the  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ -pretreated animals (group 5; Fig. 4D). Densitometric analysis is shown in Fig. 4E.

#### Suppression by $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ of OA-induced MPO, TBARS and NT elevation in lung tissue

MPO activity (Fig. 5A), a marker of inflammatory leukocyte accumulation, as well as TBARS content (Fig. 5B) and NT immunostaining (Figs. 5C–G), assumed as indicators of ROS- and PN-mediated cell injury, underwent a marked, significant increase in the lung tissue from sensitized, OA challenged guinea pigs pretreated with the vehicle (group 3) as compared with the negative controls (groups 1,2). In the sensitized guinea pigs pretreated with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 4), MPO, TBARS and, at a lesser extent, immunoreactive NT were decreased compared with the vehicle-pretreated animals (group 2), thus attaining values similar to those of the controls. On the other hand, in the guinea pigs given  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 5), MPO, TBARS and NT remained high.

#### Suppression by $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ of OA-induced decrease in MnSOD activity

As shown in Fig. 6, OA challenge of sensitized, vehicle-treated guinea pigs (group 3) caused a marked decrease in lung MnSOD activity as compared with the negative controls (groups 1,2). Pretreatment with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 4), but not with the inactive  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 5), significantly prevented the inactivation of MnSOD.

#### Suppression by $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ of $\text{PGD}_2$ in bronchoalveolar lavage

$\text{PGD}_2$ , 6keto- $\text{PG}1\alpha$  and  $\text{LTB}_4$ , are key markers for the release of pro-inflammatory prostanoids produced by activated mast cells and inflammatory leukocytes. These molecules were increased in the BAL fluid from sensitized, vehicle-treated guinea pigs challenged with OA

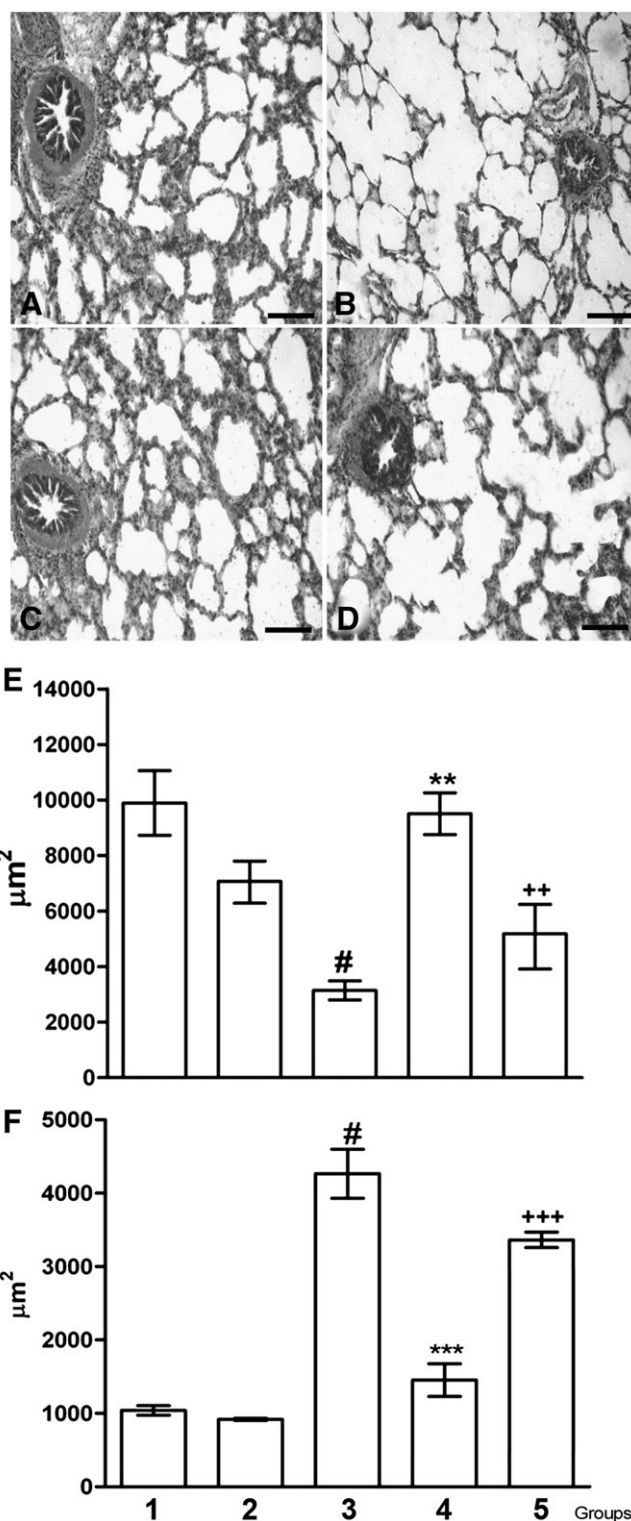


**Fig. 3.** Representative lung tissue micrographs from the different experimental groups. Compared with the naïve guinea pigs of group 1 (A), marked dilation of respiratory air spaces and constriction of bronchiolar lumen can be seen in the sensitized, vehicle-treated guinea pigs of group 3 (B). Such alterations are not visible in the  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ -pretreated guinea pigs of group 4 (C), but persist in the  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ -pretreated guinea pigs of group 5 (D). Hematoxylin and eosin. Bar = 100  $\mu\text{m}$ . Morphometric analysis of luminal area of respiratory air spaces (E) and bronchioli (F).  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 4), but not its inactive congener  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 5), suppresses bronchiolar constriction and respiratory air space dilatation, which are prominent in the sensitized, vehicle-treated animals (group 2). Significance of differences (one-way ANOVA,  $n = 6$ ): #  $p < 0.001$  vs. groups 1 & 2; \*\*\*  $p < 0.001$ , \*\*  $p < 0.01$  vs. group 3; +  $p < 0.05$ , +++  $p < 0.001$  vs. group 4.

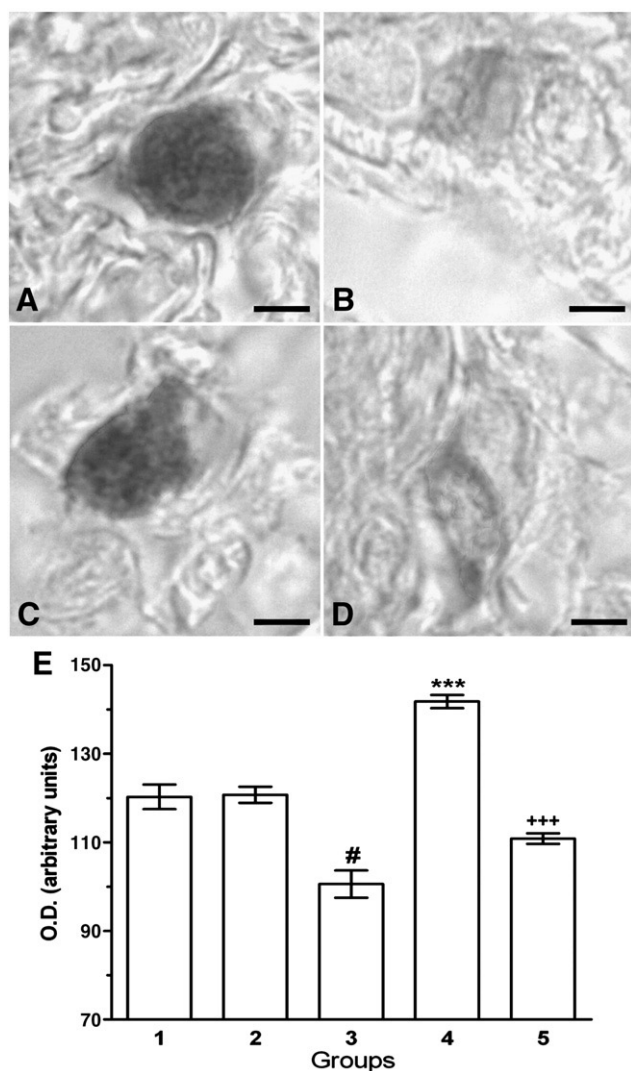


**Fig. 4.** Representative micrographs and optical density of Astra blue-stained lung mast cells in the different experimental groups: (A) naïve guinea pigs (group 1); (B) sensitized, vehicle-treated guinea pigs (group 3); (C) sensitized guinea pigs pretreated with  $Mn^{II}(Me_2DO_2A)$  (group 4); (D) sensitized guinea pigs treated with  $Zn^{II}(Me_2DO_2A)$  (group 5). (E) Densitometric analysis. Significance of differences (one-way ANOVA,  $n=6$ ): #  $p<0.001$  vs groups 1 & 2, \*\*\*  $p<0.001$  vs group 3; +++  $p<0.001$  vs. group 4.

(group 2) as compared with the negative controls (groups 1,2). In the sensitized, OA-challenged guinea pigs given  $Mn^{II}(Me_2DO_2A)$  (group 5), but not in those given  $Zn^{II}(Me_2DO_2A)$  (group 7), the levels of the above prostanoids in the BAL fluid were significantly lower than in those of the animals of group 2 (Fig. 7).

#### Suppression by $Mn^{II}(Me_2DO_2A)$ of OA-induced lung cell apoptosis

As shown in Fig. 8, OA challenge in the sensitized guinea pigs (group 3) markedly increased the activity of caspase-3, the crucial enzyme of the apoptotic cascade, as compared with the negative controls (groups 1,2), indicating that allergen-induced asthma-like reaction initiates lung cell apoptosis. Pretreatment of the animals with  $Mn^{II}(Me_2DO_2A)$  (group 4), but not with the inactive congener  $Zn^{II}(Me_2DO_2A)$  (group 5), markedly and significantly blunted caspase-3 activation.

#### Discussion

This study provides substantial evidence that the superoxide scavenger  $Mn^{II}(Me_2DO_2A)$  is able to counteract the allergen-induced

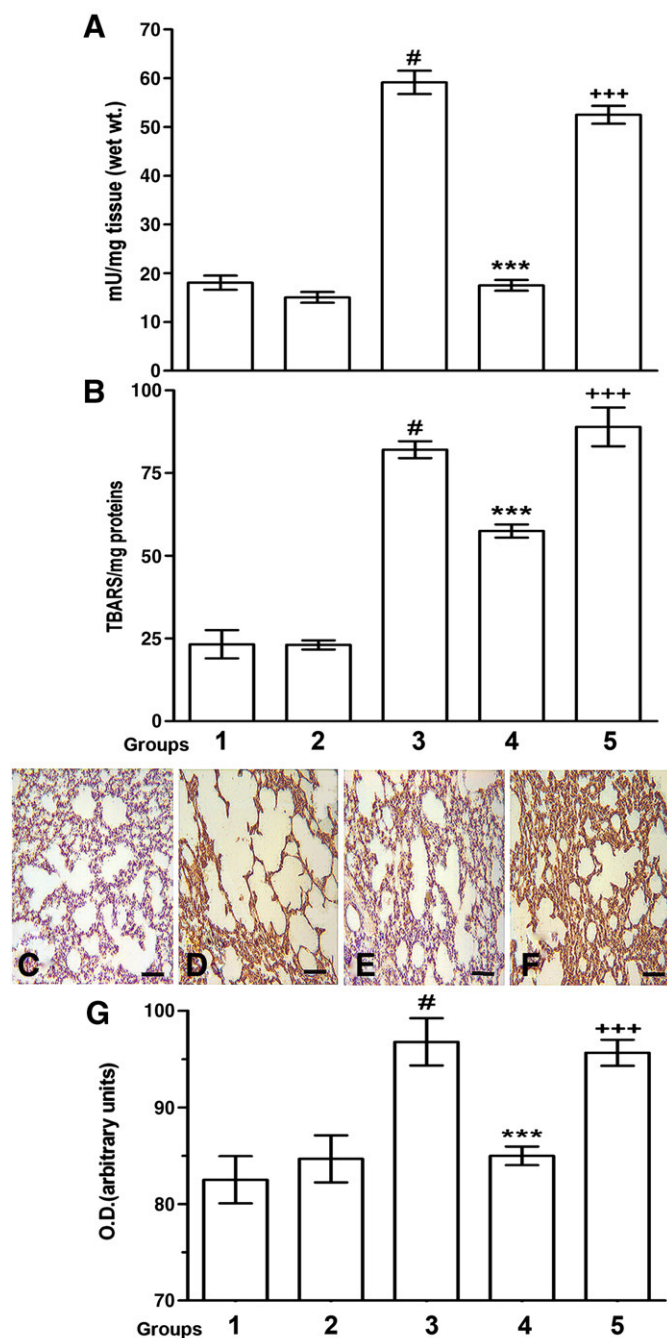


**Fig. 5.** Lung tissue MPO (A), TBARS (B) and optical density of NT immunostaining (G) in the different experimental groups. Representative micrographs of NT-immunostained lung tissue sections are from: naïve (C), sensitized vehicle-treated (D), sensitized  $Mn^{II}(Me_2DO_2A)$ -treated (E), and sensitized  $Zn^{II}(Me_2DO_2A)$ -treated (F) animals. Compared with the negative controls (groups 1,2), the noted parameters are increased in the sensitized vehicle-treated guinea pigs (group 2). Pretreatment with  $Mn^{II}(Me_2DO_2A)$  (group 4), but not with  $Zn^{II}(Me_2DO_2A)$  (group 5), decreases MPO, TBARS and the optical density of immunoreactive NT. Significance of differences (one-way ANOVA,  $n=6$ ): #  $p<0.001$  vs. groups 1 & 2; \*\*\* $p<0.001$  vs. group 3; +++  $p<0.001$  vs. group 4.

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 anti-asthmatic properties [18,36–38]. In particular,  $Mn^{II}(Me_2DO_2A)$ , administered preventatively at the 15 mg/kg dose - similar to that found to effectively decrease the acute and chronic inflammatory response in *in vivo* mouse and rat models [31, authors' unpublished data] - to OA-sensitized guinea pigs 1 h before aerosolic OA challenge caused a marked, significant suppression of

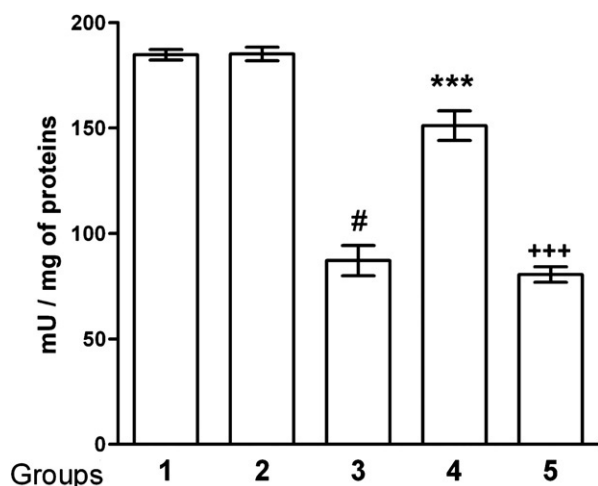


**Fig. 6.** MnSOD activity in the different experimental groups. Compared with the negative controls (groups 1,2), the activity of MnSOD is markedly decreased in the sensitized, vehicle-treated guinea pigs (group 3). Pretreatment with MnII(Me<sub>2</sub>DO<sub>2</sub>A) (group 4), but not with [ZnII(Me<sub>2</sub>DO<sub>2</sub>A)] (group 5), prevents the decay of enzyme activity. Significance of differences (one-way ANOVA, n = 6): # *p* < 0.001 vs. groups 1 & 2; \*\*\**p* < 0.001 vs. group 3; +++ *p* < 0.001 vs. group 4.

allergen-induced respiratory dysfunction (cough, dyspnea, bronchoconstriction), airway obstruction, mast cell activation, leukocyte infiltration, pro-inflammatory prostanoid release and ROS-induced lung cell injury and apoptosis as compared with the vehicle-treated controls. Moreover, MnII(Me<sub>2</sub>DO<sub>2</sub>A) is able to preserve lung MnSOD activity, which is severely impaired by oxidative stress during asthma-like reaction [11,18]. Mechanistically, the beneficial effect of MnII(Me<sub>2</sub>DO<sub>2</sub>A) appears to depend on the removal of superoxide, which specifically reacts with MnII, as the inactive congener ZnII(Me<sub>2</sub>DO<sub>2</sub>A), in which functional manganese ion was substituted with inert ZnII ion, has no significant protective effect. A preliminary study on the catalytic ability of MnII(Me<sub>2</sub>DO<sub>2</sub>A) for O<sub>2</sub><sup>•−</sup> dismutation using the cytochrome *c* assay allowed us to determine a *k*<sub>cat</sub> value of 1.1 × 10<sup>6</sup> M<sup>−1</sup> s<sup>−1</sup> (see Supplemental file 1), which is similar to that of the MnIII complex with salen, but lower than those of MnIII complexes with positively charged pyridinium-substituted meso-porphyrins, such as MnTE-2-PyP<sup>5+</sup> (Fig. 1) [45–47]. In fact, recent studies on a series of functionalized meso-porphyrins have shown that their *k*<sub>cat</sub> values are strongly influenced by electrostatic factors and increase with the electron withdrawing effect exerted by substituents [45–49]. Pyridinium groups appended to meso-porphyrins affords positively charged MnIII complex and impart a more positive redox potential to the MnIII/ MnII redox couple, increasing the SOD activity of the complexes [47]. Among these molecules, the MnIII complexes with ortho *N*-alkylpyridyl porphyrins, such as MnTE-2-PyP<sup>5+</sup>, were found to be the most active, due to the higher electron withdrawing effect exerted by ortho *N*-alkylpyridyl groups with respect to meta and para ones. Conversely, anionic substituents, such as carboxylatophenyl groups in MnTBAP<sup>3−</sup>, can lead to the formation of negatively charged complexes, reducing their dismuting ability [24,50]. Consistently, the presence of two anionic carboxylate groups in the neutral MnII(Me<sub>2</sub>DO<sub>2</sub>A) complex can be invoked to explain the lower *k*<sub>cat</sub> value observed in the present study. On the other hand, it has been recently shown that, in the case of the meta isomers of MnIII-*N*-alkylpyridylporphyrins, a high lipophilic character of the complexes can increase their bioavailability, enhancing their intracellular accumulation and compensating their lower antioxidant potency with respect to the corresponding ortho isomers [51]. This finding can contribute to explain the high antioxidant ability of MnII(Me<sub>2</sub>DO<sub>2</sub>A) observed by us *in vivo*. In fact, the two anionic carboxylate groups neutralizing the charge of MnII and the two lipophilic methyl groups make this complex capable to cross the cell membranes and attain significant intracellular concentrations [31].

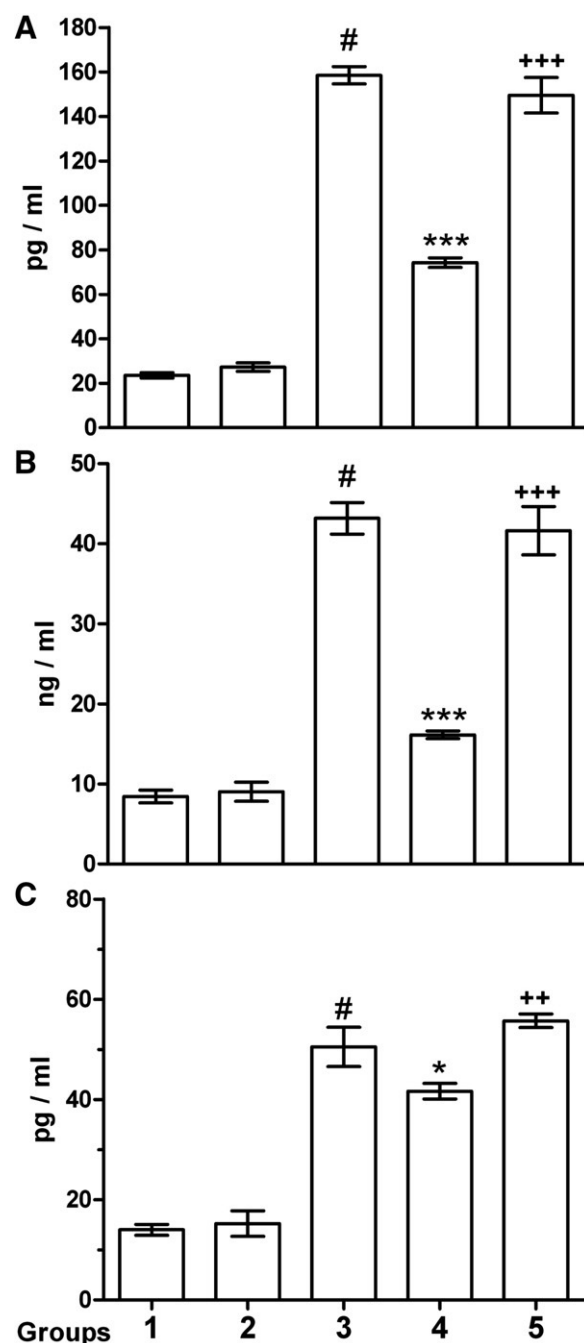


**Fig. 7.** PGD<sub>2</sub> (A), 6keto-PG1α (B) and LTB<sub>4</sub> (C), key pro-inflammatory prostanooids released by activated mast cells and inflammatory leukocytes, measured in the BAL fluid from the different experimental groups. All these molecules are increased in the sensitized, vehicle-treated guinea pigs challenged with OA (group 2) as compared with the negative controls (groups 1,2). In the sensitized, OA-challenged guinea pigs given MnII(Me<sub>2</sub>DO<sub>2</sub>A) (group 5), but not in those given ZnII(Me<sub>2</sub>DO<sub>2</sub>A) (group 7), the levels of these prostanooids were significantly lower than in the animals of group 2 (Fig. 7). Significance of differences (one-way ANOVA, n = 3): # *p* < 0.001 vs. groups 1 & 2; \*\*\**p* < 0.001, \* *p* < 0.05 vs. group 3; ++ *p* < 0.01, +++ *p* < 0.001 vs. group 4.

An investigation of the redox properties of MnII(Me<sub>2</sub>DO<sub>2</sub>A) showed that the complex undergoes only a one-electron oxidation step, which can be attributed to the MnII → MnIII process [31]. This lead us to suggest that the scavenging ability of this complex is due to the MnII/MnIII redox couple, as generally proposed for MnII complexes with pentaazamacrocycles [6]. Interestingly, the *E*<sub>1/2</sub> potential for the MnII/MnIII couple is higher for MnII(Me<sub>2</sub>DO<sub>2</sub>A) than for the MnII complexes with the tri- and tetracarboxylate ligands HEDTA<sup>3−</sup> and EGTA<sup>4−</sup>. These latter afford the negatively charged complexes

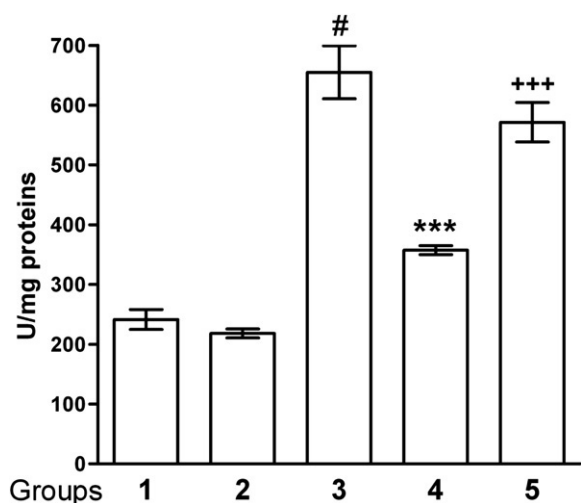


**Fig. 8.** Caspase-3 activity, assumed as the marker of lung cell apoptosis, in the different experimental groups. Compared with the negative controls (groups 1,2), the activity of caspase-3 is markedly increased in the sensitized, vehicle-treated guinea pigs (group 3). Pretreatment with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 4), but not with  $\text{Zn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  (group 5), prevents the increase in enzyme activity. Significance of differences (one-way ANOVA,  $n = 6$ ): #  $p < 0.001$  vs. groups 1 & 2; \*\*\* $p < 0.001$  vs. group 3; +++  $p < 0.001$  vs. group 4.

$\text{Mn}^{\text{II}}(\text{HEDTA})^-$  and  $\text{Mn}^{\text{II}}(\text{EGTA})^{2-}$ , which showed a much lower antioxidant ability than the neutral complex  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  [31], in keeping with the suggestion that electrostatic factors may play an important role in determining the antioxidant properties of these compounds [52]. It should be underscored that different redox couples, involving higher oxidation states of manganese generated by the strong oxidant action of PN, could also participate in the antioxidant action of  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  in biological environments [31]. Therefore, we cannot rule out that, besides superoxide,  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  may also scavenge and inactivate PN. In fact, in the inflammatory process, superoxide and PN can be viewed as the two aspects of the same medal, as both are generated during inflammatory reaction and contribute to cell and tissue injury [53]. Should  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  be proven to also function as a peroxynitrite decomposition catalyst, this would be a further issue of interest in this compound from a mechanistic perspective.

The present findings provide additional evidence for the involvement of  $\text{O}_2^-$  in the pathophysiology of asthma, as previously reported [2–4], and support the concept that substances capable to decrease  $\text{O}_2^-$  levels may be promising therapeutic tools for this disease [16–18]. Of note, the present study also fits well with the recent observation that transgenic mice with elevated levels of Cu/Zn SOD in the lungs are resistant to allergen-induced asthma-like response [54].

In asthmatic patients, an inverse correlation has been found between superoxide production and  $\text{FEV}_1$ , suggesting a close relationship between airway obstruction and increased generation of superoxide by inflammatory cells in the lungs [55]. In the present study, we have observed that bronchoconstriction and leukocyte infiltration, evaluated as lung MPO activity, were significantly decreased in the guinea pigs treated with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ . Of note, leukocyte infiltration is a key histopathological hallmark of the asthmatic airways: recruitment of leukocytes and the release of inflammatory mediators, such as prostanoids and cytokines, by these cells are prominent pathogenic features of the allergic response [56]. Therefore, the decrease in leukocyte infiltration may be a key mechanism for the noted beneficial effect of  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$ . This notion fits well with the anti-inflammatory properties of Mn-SOD and synthetic SOD-mimetic antioxidant compounds successfully used as therapeutics for lung fibrosis in animal models and in humans [6]. Besides leukocyte infiltration, pretreatment with  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  also

prevented mast cell activation, which is also sparked by superoxide [57,58]. Mast cell mediators, such as histamine, cause bronchoconstriction and smooth muscle cell proliferation and recruit further inflammatory cells, thereby initiating a vicious circle that may amplify allergen-dependent bronchoconstriction, lung injury and airway remodeling and favour the transition from acute to chronic asthma [15].

In the present guinea pig model,  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  affords protection against allergen-induced asthma-like reaction similarly to native MnSOD [17] and the previously studied SOD-mimetic compounds [18], both capable of catalyzing superoxide dismutation. Based on the chemical similarities with the latter compounds,  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  may also conceivably function as a catalytic antioxidant with SOD activity. Compared with these molecules, composed by an organic macrocyclic pentadentate scaffold chelating a  $\text{Mn}^{\text{II}}$  ion [17],  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  would have the advantage of being a smaller, lipophilic molecule with better pharmacokinetic profile, being capable to readily enter the cells [31] and, conceivably, dismutate superoxide at the cytoplasmic sites of generation.

In conclusion, the present findings strongly support the concept that  $\text{Mn}^{\text{II}}(\text{Me}_2\text{DO}_2\text{A})$  and, in general,  $\text{O}_2^-$  dismutation catalysts may represent novel, promising therapeutic tools for the treatment of asthma, as well as of other bronchopulmonary diseases characterised by ROS over-production and oxidative stress.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.freeradbiomed.2010.02.041.

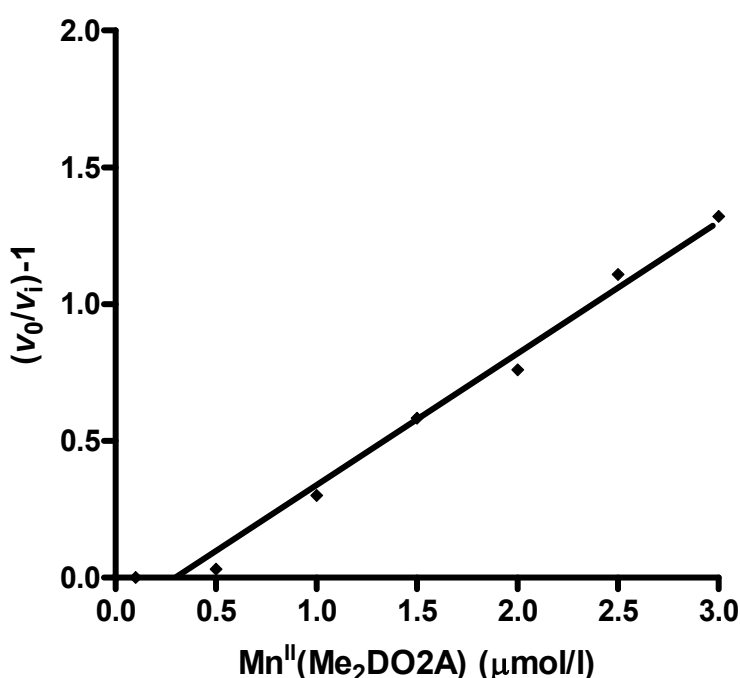
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## Supplemental file # 1

**Determination of  $O_2^{\bullet-}$  catalytic rate constant of  $Mn^{II}(Me_2DO_2A)$ .** The rate constant for the reaction of the  $Mn^{II}(Me_2DO_2A)$  with  $O_2^{\bullet-}$  was determined by competition kinetics using cytochrome c, following the previously described method [1,2]. Briefly, micromolar serial dilutions of  $Mn^{II}(Me_2DO_2A)$  were used. Inhibition of 10  $\mu M$  cytochrome c reduction by the compound, plotted as  $\{(v_0/v_i) - 1\}$  vs. the concentration of  $Mn^{II}(Me_2DO_2A)$ , yielded a straight line, as shown in the figure. The  $v_0$  was the uninhibited rate of reduction of cytochrome c by  $O_2^{\bullet-}$  and  $v_i$  the rate of cytochrome c reduction inhibited by the different concentrations of the compound. From the plot, the concentration that causes 50% inhibition of cytochrome c reduction by  $O_2^{\bullet-}$  (1 unit of activity) was found at  $(v_0/v_i) - 1$ ,  $IC_{50} = 2.4 \times 10^{-6}$  M. On the basis of the competition with 10  $\mu M$  cytochrome c [2], at 50% inhibition, the rates of the reactions of cytochrome c and the compound with  $O_2^{\bullet-}$  are equal, i.e.,  $K_{cat} [Mn^{II}(Me_2DO_2A)] = K_{cyt} [cytochrome\ c]$ , where  $K_{cyt} = 2.6 \times 10^5\ M^{-1}\ s^{-1}$ . Hence, the catalytic rate constant was calculated to be  $K_{cat} = 1.1 \times 10^6\ M^{-1}\ s^{-1}$ .



Plot of  $\{(v_0/v_i) - 1\}$  vs.  $Mn^{II}(Me_2DO_2A)$  concentrations, where  $v_0$  is the rate of reduction of 10  $\mu M$  cytochrome c by  $O_2^{\bullet-}$  and  $v_i$  the rate of reduction of cytochrome c inhibited by the catalyst. The reaction was performed at 25 °C in 3 mL 0.05 M Tris buffer, pH 7.4, containing 40  $\mu M$  xanthine and 10 nM xanthine oxidase [2].

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