

Influence of MnTE-2-PyP on Inflammation and Lipid Peroxidation in Mouse Asthma Model

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ABSTRACT

Our aim was to investigate the effects of MnTE-2-PyP on some markers of inflammation and lipid peroxidation in mouse asthma model. 24 female mice were divided into four groups: group 1, controls; group 2, injected with ovalbumin (OVA); group 3, treated with MnTE-2-PyP; and group 4, treated with ovalbumin and MnTE-2-PyP. The mice from groups 2 and 4 were injected with 10 µg OVA and 1 mg Imject Alum[®] in 100 µL phosphate buffered saline (PBS) on days 0 and 14. The animals from groups 1 and 3 were injected with 100 µL PBS + Imject Alum[®] (1:1). The animals from groups 2 and 4 were subjected to a 30 min aerosol challenge of 1% ovalbumin on days 24, 25 and 26 and those from groups 1 and 3 were subjected to aerosol challenge of PBS at the same time and duration. One hour before inhalation, and 12 hours later the animals from groups 3 and 4 were injected with 100 µL MnTE-2-PyP solution in PBS containing 5 mg/kg. The total cell number, total protein content and 8-isoprostane, IL-4 and IL-5 levels in the bronchoalveolar lavage fluid increased in group 2 as compared to the control group. Malonaldehyde content in the lung homogenate and IgE levels in the serum also increased in this group. The total cell number, total protein content, and levels of 8-isoprostane, IL-4, IL-5 and IgE decreased significantly in group 4 as compared to the OVA group. The parameters set out above in group 3 did not differ significantly from those of the control group. MnTE-2-PyP administered intraperitoneally, 48 hours after the last nebulization, reduced the inflammation and lipid peroxidation in mouse asthma model.

Keywords: Asthma; Inflammation; Interleukins; 8-Isoprostane; Lipid Peroxidation; MnTE-2-PyP

1. Introduction

Asthma is a lung disease characterized by airspace inflammation and oxidative stress [1-4]. Elevated levels of reactive oxygen species (ROS), released by inflammatory cells, either directly or through the formation of products of lipid peroxidation, play a role in enhancing the inflammatory response in these diseases. The presence of oxidative stress is important in the pathogenesis, severity and treatment of asthma [5]. Increasing evidence suggests that abnormalities in mitochondria are involved in several mitochondrial diseases, but also in the development of asthma [6,7]. Recently, antioxidants to prevent and to treat mitochondria in patients with mitochondrial diseases, including asthma, has received much attention, especially because antioxidant approaches seem to have few or no adverse effects [8]. Different classes of antioxidants are known. Among them, the group of catalytic

manganese metalloporphyrins takes center stage with their accumulation into mitochondria. They have at least four antioxidant properties, such as removal of superoxide (O_2^-), hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), and lipoperoxides [9,10]. Based on this information, we set the goal to investigate the effects of MnTE-2-PyP (Manganese(III) 5, 10, 15, 20-tetrakis (*N*-ethylpyridinium-2-yl)porphyrin), a manganese-*meso*porphyrin also known as AEOL-10113, on markers of inflammation and lipid peroxidation in a mouse ovalbumin (OVA) sensitization model of asthma [11].

2. Materials and Methods

2.1. Chemicals

Ovalbumin, grade V, and phosphate buffered saline (PBS), were purchased from Sigma-Aldrich Company, Nitrocellulose filters with 5 µm pores were from Milli-

pore Corp, IL-4 and 5 ELISA Kits were from R&D Systems, 8-Isoprostane EIA Kit was from Cayman chemicals, Mouse IgE ELISA Sets were purchased from BD Biosciences, and the Imject Alum[®] was from the Pierce Chemical Company (USA). MnTE-2-PyP was kindly provided by Prof. Ines Batinić-Haberle from the Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina, USA.

2.2. Animals and Experimental Protocol

The experiment was performed in accordance with Animal Welfare Regulations and was approved by the University Ethics Committee.

The study was carried out on 24 female C57Bl/6 mice (weight 20 ± 2 g, 8 - 10 week old). The animals were raised at the University vivarium at a temperature of $22^\circ\text{C} \pm 2^\circ\text{C}$ and humidity of $50\% \pm 10\%$, and were given a normal pelleted diet and water *ad libitum*. The mice were divided into four groups: group 1, controls; group 2, injected with ovalbumin; group 3, treated with MnTE-2-PyP and group 4, treated with OVA and MnTE-2-PyP. Airway inflammation was induced by OVA immunization and challenge. The animals from groups 1 and 3 were injected *i.p.* with a 100 μL phosphate-buffered saline (PBS) + Imject AlumR (1:1) on days 0 and 14. The animals from groups 2 and 4 were injected with a 100 μL ovalbumin solution, containing 20 μg OVA on the same days. On days 24, 25 and 26, mice from groups 1 and 3 were given inhalation with PBS for 30 min, and those from groups 2 and 4 were given inhalation with 1% ovalbumin solution (OVA dissolved in PBS). For this purpose, a special plexiglass chamber was used. One hour before inhalation, and 12 hours later the animals from groups 1 and 2 were injected *i.p.* with 100 μL PBS, and those from groups 3 and 4 received a 100 μL MnTE-2-Pyp dissolved in PBS, containing 5 mg/kg, that is, the total daily dose was 10 mg/kg.

The solution was sterilized by filtration through 0.2 μm filters.

2.3. Bronchoalveolar Lavage Fluid (BALF)

To obtain BALF, the animals were sacrificed on day 28 (48 hours after the last inhalation) under thiopental anaesthesia (50 mg/kg). The chest was opened and the lungs were perfused *in situ* via the right heart ventricle with saline (10 mL). Triple lavage of the left lung through the trachea was performed with a total volume of 2.5 mL of saline. The right lung was ligated at the hilum, cut and then removed from the chest and used to prepare the lung homogenate.

Cytological, Biochemical and Immunological Assays of BALF

One aliquot of the BALF was used for the purpose of

total cell number $\times 10^5/\text{mL}$. The cells were then removed by centrifugation at $300 \times g$ for 10 min. The supernatant of BALF was used to measure interleukins and 8-isoprostane levels. The cell pellet was resuspended in 0.5 mL of saline, and differential cell count using Millipore filters by the method of Danos and Keebler, modified by Saltini [12] was performed. The total protein content in ng/mL by the method of Lowry *et al.* [13], the levels of IL-4 and IL-5 in pg/mL by the ELISA method, and the level of 8-isoprostane in ng/mL by the ELISA method in accordance with manufacturer's instructions, were investigated in the supernatant of BALF.

2.4. Biochemical Assays of Lung Homogenate

Lung homogenate was obtained from the right lung. The tissue was homogenized with potassium chloride (KCl) in 1:10 ratio (lung mass by KCl solution volume). The homogenate was centrifuged ($9000 \times g$, 30 min), and the supernatant was stored on ice. Malonaldehyde (MDA) content in nmol/g was measured by the method of Ohkawa *et al.* [14].

2.5. Immunological Assay of Serum

Blood was drawn from the abdominal aorta by using vacuum blood collection tubes. The blood was allowed to clot for 30 minutes, and then centrifuged at $1000 \times g$ for 10 min to achieve serum separation. The samples were kept frozen at -20°C until serum IgE analysis in ng/mL was made by the ELISA method in accordance with manufacturer's instructions.

2.6. Statistical Analysis

Experimental data were analyzed using SPSS 14. When we tested for normality, one variable-MDA showed non-parametric distribution, and we used medians, interquartile range and Mann-Whitney test for comparison. For the rest of the variable we applied post-hoc ANOVA test and data were presented as mean \pm standard error of mean (SEM). $P < 0.05$ was considered statistically significant.

3. Results

The total cell number in group 2 (OVA-sensitized mice) increased more than four fold in BALF (479% as compared to the controls, $P = 0.025$). The increase of this parameter in group 4 (OVA + MnTE-2-PyP) was significantly lower (178%) than that in group 2. The eosinophil percentage was 28% in group 2 and 18% in group 4, versus 0.5% in the control group (**Table 1**). The total protein content in group 2 showed the same dynamics (**Table 1**). The levels of IL-4 and IL-5 increased sharply in group 2 (OVA) up to 1849% ($P = 0.0006$) and 350% ($P = 0.016$) respectively, in comparison with the control

Table 1. Effect of MnTE-2-PyP on markers of inflammation in BALF and lipid peroxidation in lung homogenate in mouse model of asthma.

Parameters	28 day after treatment (48 hours after the last inhalation)				
	Group	Control	OVA	MnTE-2-PyP	OVA + MnTE-2-PyP
Total cell number in BALF ($\times 10^5$ mL)		2.26 \pm 0.09	10.84 \pm 2.34*	4.00 \pm 0.40	4.04 \pm 0.41 [†]
AMas (%)		90.2	58.8	85.3	70.0
PMN (%)		4.6	8.2	5.5	5.8
Eo (%)		0.5	28.0	2.5	18.0
Total protein content (mg/mL) mean \pm SEM		0.454 \pm 0.025	0.563 \pm 0.023*	0.419 \pm 0.038	0.463 \pm 0.050 [†]
MDA content (n mol/g) Median		22.86	42.7*	22.68	20.86 [†]
Min - max		21.56 - 26.88	39.2 - 46.20	15.12 - 32.48	19.04 - 22.68
Q ₃ - Q ₁		2.66	6.8	17.36	3.64

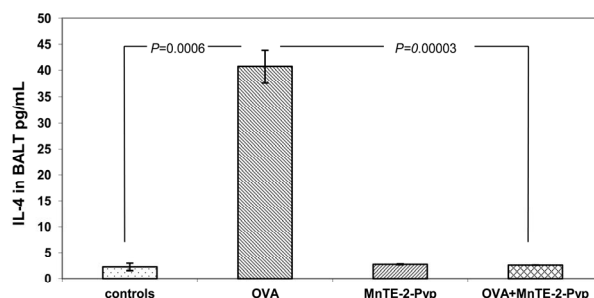
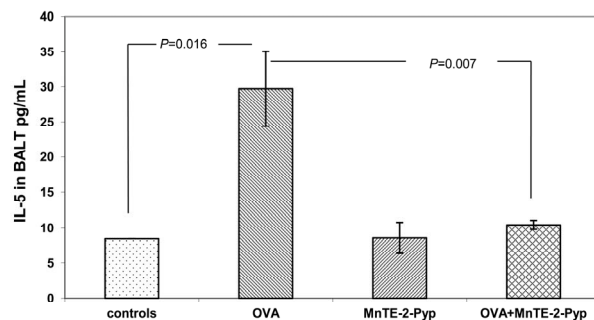
Abbreviations: OVA, ovalbumin; MnTE-2-PyP, Manganese(III) 5, 10, 15, 20-tetrakis(N-ethylpyridinium-2-yl)porphyrin; AMas, alveolar macrophages; PMN, polymorphonuclear leukocytes; Eo, eosinophils; IL-4 and IL-5, interleukins 4 and 5; IgE, immunoglobulin E; MDA, malone dialdehyde; SEM, standart error of mean; Q₃ - Q₁, interquartile range. *: Different from control at $P < 0.05$; [†]: Different from group 2 (OVA) at $P < 0.05$.

group (Figures 1 and 2). OVA and antioxidant treatment (group 4) decreased significantly these parameters as compared to group 2 (OVA), up to 115% ($P = 0.00003$) and 123% ($P = 0.007$) respectively. The IgE levels in the group treated with OVA alone elevated up to 340% as compared to the control animals ($P = 0.00008$). The increase in group 4 (OVA + MnTE-2-PyP) was significantly lower (264%, $P = 0.049$) as compared to group 2 (Figure 3). The changes in 8-isoprostane levels in BALF and MDA content in lung homogenate, which are markers of lipid peroxidation, followed the same trend among the four groups. The level of 8-isoprostane in group 2 was three times higher than that in the control group ($P = 0.03$), whereas in group 4 (treated with OVA and MnTE-2-PyP) it was significantly lower than that in group 2, and the values were approximate to those of the controls (Figure 4). The MDA content increased up to 187% in group 2 as compared to the control group. In group 4 this content was approximate to that in the controls, and was lower as compared to group 2 ($P = 0.03$), (Table 1). The levels of interleukins and IgE as well as 8-isoprostane in group 3 (MnTE-2-PyP alone) did not differ significantly from those of control group.

4. Discussion

The experimental data of our study showed that the MnTE-2-PyP has a beneficial effect on the indicators of inflammation and lipid peroxidation. Metaloporphyrins are a new and potent class of lipid peroxidation inhibitors. Their potency is connected not only to their redox potential, but also to other factors that can contribute to their ability to act as electron acceptors. MnTE-2-PyP is very effective in the elimination of reactive species, particularly O_2^- and $ONOO^-$, with the highest rate constant

among the other synthetic antioxidants [15-18]. Manganese(III) 5, 10, 15, 20-tetrakis(N-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP) and Manganese(III) 5, 10, 15, 20-tetrakis(N-hexylpyridinium-2-yl)porphyrin (MnTnHex-2-PyP) have an excellent SOD activity *in vitro* and *in vivo* [19,20]. The biological role of MnTBAP and MnTE-2-PyP is related to their peroxynitrite-binding activity and reduction of the carbonate radical. Logcat

**Figure 1.** The level of IL-4 in BALF. Each point represents the mean \pm SEM for six mice.**Figure 2.** The level of IL-5 in BALF. Each point represents the mean \pm SEM for six mice.

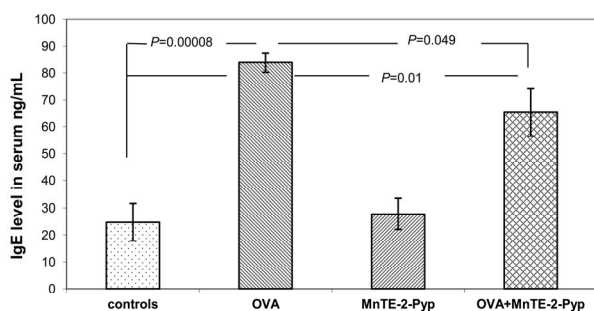


Figure 3. The level of IgE in serum. Each point represents the mean \pm SEM for six mice.

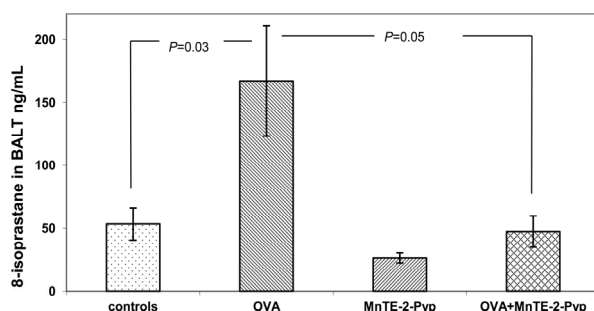


Figure 4. The level of 8-isoprostane in BALF. Each point represents the mean \pm SEM for six mice.

(O_2^-) of MnTBAP is about 3.16, which is about 5 - 6 times less than the SOD activity of the powerful SOD mimetic MnTE-2-PyP and CuZn SOD. Positively charged MnTE-2-PyP and related analogues are very suitable for SOD mimetics and $ONOO^-/CO_3^-$ cleaners. MnTE-2-PyP has a potent catalytic antioxidant-like effect of extracellular superoxide dismutase. The strong porphyrin-based compounds are based on a "structure-activity" relationship, such as Mn(III) *meso*-tetraakis (*N*-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP) and its hexyl analogue (MnTnHex-2-PyP) [21].

We applied antioxidants in a dose of 10 mg/kg/daily, divided into doses over 12 hours, starting with the assertion that good tolerance in mice is observed at two doses of 15 mg/kg/day and the plasma half-life varies considerably. This is the most commonly used (as single or multiple) therapeutic dose of this porphyrin [22]. MnTE-2-PyP administered *i.p.* or orally reaches maximum concentration in plasma in 0.33 hours [16,17]. It accumulates at high levels in liver, kidney, and spleen, at moderate levels in lungs and heart, and at low levels in brain. The plasma half-life for a single *i.p.* dose of 10 mg/kg in mice is about 1 hour and the half-life in the body is significantly longer ranging from 60 - 135 hours [22-24]. Studies reveal that MnTBAP and MnTM-4-PyP are effective in animal models of oxidative stress by forming superoxide [25-27], hydrogen peroxide [28,29] and peroxynitrite [30,31]. Many water-soluble *meso* substituted man-

ganese porphyrins have a molecular weight above 800, quickly pass through cell membranes and are distributed in mitochondria [23]. MnTE-2-PyP in the liver cells showed slightly higher accumulation in the mitochondria, as compared with the cytosol (Spasojevich I., unpublished data).

This compound not only decreases the primary insult of reactive species to biological molecules, but also inhibits the activation of transcription factors which in turn leads to suppression of expression of those cytokines and enzymes that perpetuate secondary oxidative stress [20,32]. According to Gauter-Fleckenstein *et al.* [33] the inhabitation of excessive cellular activity takes place through the suppression of the transcriptional activity, particularly suppressing HIF-1 α (hypoxia inducible factor 1 α) activation in a long-lasting effect. Therefore reducing the total number of cells, the amount of total protein observed after treatment with mangan porphyrin can be explained by the ability of the antioxidants to inhibit the expression of VCAM-1 (vascular cell adhesion molecule-1) responsible for the accumulation of inflammatory cells, and thus to decrease airway hyperreactivity [34]. After intratracheal introduction, the antioxidant dramatically reduces the severity of airway inflammation in the airways in OVA-induced murine asthma. The reduction in the number of eosinophils, neutrophils and lymphocytes in BALF is more than 80% [34-36]. VCAM-1 and ICAM-1 (intra-cellular adhesion molecule) participate in the migration of eosinophils and neutrophils and contribute to eosinophilic inflammation in animal models. MnTE-2-PyP has been shown to alter cell signaling and reduce inflammation by reducing NF- κ B activity [37]. Piganelli *et al.* showed that MnTE-2-PyP inhibits T cell proliferation, while lipopolysaccharide-stimulated macrophages treated with the compound, inhibit TNF- α (tumor necrosis factor- α) and NADPH release of superoxide [38]. The beneficial effects of Mn alkylpyridylporphyrins have been observed in various diseases, associated with oxidative stress, such as radiation injury, Alzheimer's disease, cancer, diabetes, central nervous system injuries, ischemia/reperfusion conditions, pulmonary emphysema, and other diseases [19,33,38-43].

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