**ABSTRACT**

Background and objective: Investigation of the effects of MnTnHex-2-PyP on some markers of inflammation and lipid peroxidation in an asthma mice model.

Methods: The experiment was carried out on 24 female mice C57Bl/6, divided into four groups: group 1, controls; group 2, injected with ovalbumin (OVA); group 3, treated with MnTnHex-2-PyP and group 4, treated with OVA and MnTnHex-2-PyP. The animals from groups 1 and 3 were injected i.p. on days 0 and 14 with a 100 $\mu$l phosphate-buffered saline (PBS), and those from groups 2 and 4 were injected with a 100 $\mu$l ovalbumin solution, containing 20 $\mu$g OVA. On days 24, 25 and 26 the mice from groups 1 and 2 were inhaled with PBS for 30 min, and those from groups 2 and 4 were given a 1% ovalbumin solution. One hour before inhalation, and 12 hours later the animals from groups 1 and 2 were injected i.p. with 100 $\mu$l PBS, and those from groups 3 and 4 received a 100 $\mu$l MnTnHex-2-Pyp solution in PBS containing 0.05mg/kg.

Results: Ovalbumin alone (group 2) increased the total cell number, total protein content, the levels of IL-4, IL-5 and 8-isoprostane in bronchoalveolar lavage. Elevations were observed in IgE level in serum, and the malone dialdehyde (MDA) content in the lung homogenate. These markers were decreased significantly in group 4 as compared to the OVA group.

Conclusions: MnTnHex-2-Pyp reduces the inflammation and lipid peroxidation in Ovalbumin-induced mice asthma model.

**JEL CLASSIFICATION & KEYWORDS**

I19 Asthma BALF Inflammation Lipid Peroxidation Lung Homogenate MnTnHex-2-PyP.

**INTRODUCTION**

The large surface area, blood supply and high oxygen environment predispose the lungs to cellular injury induced by oxidative stress (1). It is widely agreed that a link exists between oxidants and their effect on a number of pulmonary diseases, particularly asthma pathogenesis (2-5). The incidence of bronchial asthma, a chronic inflammatory disease, has reached more than 25% in recent years in the West European countries (6). It is assumed that the pathogenesis of asthma is associated with chronic airway inflammation and increased oxidative stress (7,8). Inflammatory cells such as activated eosinophils, neutrophils, monocytes and macrophages, by way of infiltrating the airways in asthmatics, have the exceptional capacity to generate reactive oxygen species (ROS) and lipid peroxidation products under the influence of various stimuli (9,10). The body has a powerful antioxidant system, which may delay or prevent oxidation, but also eliminate reactive oxygen species. At high levels of oxidative stress, however, antioxidants become depleted, and an imbalance between oxidants and antioxidants occurs, which causes pathological damage (11). In recent years new different classes of antioxidants have been introduced as a method for treatment of pulmonary diseases associated with oxidative stress. Catalytic manganese metalloporphyrins belong to a novel and potent class of lipid peroxidation inhibitors. We aimed to study the effect of MnTnHex-2-Pyp (Manganese (III) 5,10,15,20-tetrakis(N-hexylpyridinium-2-yl) porphyrin) on some markers of inflammation and lipid peroxidation in asthma mice model.

**Materials and methods**

- **Chemicals**

Ovalbimin, grade V and phosphate buffered saline (PBS), were purchased from the Sigma-Aldrich Company. Nitrocellulose filters with 5$\mu$m pores were received from Millipore Corp, IL-4 and IL-5 ELISA Kits were purchased from R&D Systems, 8-Isoprostan EIA Kit received from Cayman chemicals, Mouse IgE ELISA Set was purchased from BD Biosciences, and Immulon Alum® was obtained from Pierce Chemical Company (USA).

MnTnHex-2-Pyp was kindly provided by Ines Batinić-Haberle from the Department of Radiation Oncology, Duke University Medical Center, Durham, North Carolina, USA.

- **Animals and experimental protocol**

The experiment was performed in accordance with the regulations for animal welfare and was approved by the University Ethics Committee.

The study was carried out on 24 female mice C57Bl/6 (weight 20+2g, 8-10 weeks old). The animals were raised at the University animal vivarium at a temperature of 22+2°C and humidity of 50+10%, given normal pelleted diet and water ad
Effects of MnTnHex-2-PyP on markers of inflammation and lipid peroxidation in asthma mice model

The mice were divided into four groups: group 1, controls; group 2, injected with ovalbumin (OVA); group 3, treated with MnTnHex-2-PyP and group 4, treated with OVA and MnTnHex-2-PyP. The animals from groups 1 and 3 were injected intraperitoneally (i.p.) on days 0 and 14 with 100 μl PBS+Imject Alum® (1:1). The animals from groups 2 and 4 were injected with 100 μl ovalbumin solution containing 20 μg OVA (p0012-protocol). On days 24, 25 and 26 the mice from groups 1 and 2 were inhaled with PBS for 30 min, and those from groups 2 and 4 were given a 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 (0.05mg/kg) MnTnHex-2-PyP dissolved in PBS. The solution was sterilized by filtration through 0.2 μm filters. For all injections, individual sterile needles were used.

- Bronchoalveolar lavage fluid (BALF)
  To obtain BALF, the animals were sacrificed on day 28 (48 hours after the last inhalation) by exsanguination using vacuum blood collection tubes. 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 with a total volume of 2.5 mL of saline was performed. The right lung was ligated at the hilus, 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 a total cell number x 10⁵/L. The cells were then removed by centrifugation at 300 x g for 10 min. The supernatant of BALF was used for the measurement of interleukins and 8-isoprostane levels. The cell pellet was resuspended in 0.5 mL of saline, and differential cell count was performed with Milipore filters by the method of Danos and Keebler, modified by Saltini (12). 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 the manufacturer's instructions, were investigated in the supernatant.

- Biochemical assays in lung homogenate
  Lung homogenate was obtained from the right lung. The tissue was homogenized with KCl in 1:10 ratio. The homogenate was centrifuged (9000 x g, 30 min) and the supernatant was stored on ice. MDA content in nmol/g was measured by the method of Ohkawa et al.(14).

- Immunological assay of serum
  The blood was allowed to clot for 30 minutes, and then it was centrifuged at 1000 x g for 10 min for serum separation. Samples were stored on ice at -20°C until analyzed for IgE levels in the serum. IgE in ng/mL was investigated by the ELISA method in accordance with the manufacturer's instructions.

- Statistical analysis
  Experimental data were analyzed statistically by one-way analysis of variance (ANOVA). In the table the results were presented as means±SEM. A value of p<0.05 was considered statistically significant. The statistical procedure was performed with Statgraphics plus for Windows 5.0.

Results
The total cell number in group 2 (sensitized and inhaled with OVA) increased up to 461% (p=0.025) as compared with the control group. In group 4 (treated with OVA and MnTnHex-2-PyP) this increase was significantly lower (145%), (p=0.04) as compared to group 2 (Table 1, Fig. 1). The eosinophil percentage was elevated up to 28% in group 2, but this same parameter increased up to 15.2% in group 4.

Table 1: Effect of MnTnHex-2-PyP on markers of inflammation and lipid peroxidation in BALF, serum and lung homogenate in mice asthma model

<table>
<thead>
<tr>
<th>Parameters/Groups</th>
<th>Control</th>
<th>OVA</th>
<th>MnTnHex-2-PyP</th>
<th>OVA+ MnTnHex-2-PyP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell number in BALF (x 10⁵/mL)</td>
<td>2.26±0.18</td>
<td>10.84±6.2*</td>
<td>3.27±0.19</td>
<td>3.41±0.41†</td>
</tr>
<tr>
<td>AMas (%)</td>
<td>90.2</td>
<td>58.8</td>
<td>87.1</td>
<td>76.9</td>
</tr>
<tr>
<td>PMN (%)</td>
<td>4.6</td>
<td>8.2</td>
<td>4</td>
<td>3.1</td>
</tr>
<tr>
<td>Eo (%)</td>
<td>0.5</td>
<td>28</td>
<td>2.2</td>
<td>15.2</td>
</tr>
<tr>
<td>Total protein content (mg/mL)</td>
<td>0.45±0.035</td>
<td>0.56±0.033</td>
<td>0.44±0.100</td>
<td>0.45±0.040</td>
</tr>
<tr>
<td>IL-4 (pg/mL)</td>
<td>2.20±0.70</td>
<td>40.68±3.21†</td>
<td>2.62±0.03</td>
<td>2.61±0.03†</td>
</tr>
<tr>
<td>IL-5 (pg/mL)</td>
<td>8.47±0.0005</td>
<td>29.72±5.33*</td>
<td>5.73±0.50</td>
<td>15.09±1.58*</td>
</tr>
<tr>
<td>IgE (ng/mL)</td>
<td>24.68±6.91</td>
<td>83.86±3.62*</td>
<td>26.63±11.15</td>
<td>46.34±11.04†</td>
</tr>
<tr>
<td>8-isoprostane (ng/mL)</td>
<td>53.24±23.03</td>
<td>166.92±44.05*</td>
<td>34.53±7.69</td>
<td>45.34±20.54†</td>
</tr>
<tr>
<td>MDA content (nmol/g)</td>
<td>23.45±1.17</td>
<td>42.70±3.50*</td>
<td>20.02±1.54</td>
<td>21.70±0.98†</td>
</tr>
</tbody>
</table>

Abbreviations: OVA, ovalbumin; MnTnHex-2-PyP, Manganese (III) 5,10,15,20-tetrakis(N-hexylpyridinium-2-yl) porphyrin; AMas, alveolar macrophages; PMN, polymorphonuclear leukocytes; Eo, eosinophile; IgE, immunoglobulin E; MDA, malone dialdehyde
* Different from control at p<0.05 by analysis of variance
† Different from group 2 (OVA) at p<0.05 by analysis of variance

Source: Author

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EFFECTS OF MnTnHex-2-PyP ON MARKERS OF INFLAMMATION AND LIPID PEROXIDATION IN ASTHMA MICE MODEL

Figure 1: Total cell number in BALF x 10^5 (Each point represents the mean±SD for six mice.)

Source: Author

Figure 2: The level of IL-4 in BALF. (Each point represents the mean±SD for six mice.)

Source: Author

Figure 3: The level of IL-5 in BALF.

Source: Author

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The increase in group 4 (OVA+MnTnHex-2-PyP) was significantly lower (188%, p=0.036) as compared to group 2 (Table 1, Fig. 4).

The markers of lipid peroxidation, 8-isoprostane and MDA content, in BALF and lung homogenate showed the same changes. The level of 8-isoprostane in the group with OVA was three times higher than that in the control group (p=0.05), whereas in group 4 (treated with MnTnHex-2-PyP and OVA) was significantly lower than that in group 2 (p=0.05), (Table 1, Fig. 5). The MDA content increased in group 2 up to 181% as compared with the control group. In group 4 the value of this content was approximate to that in the control, and was significantly lower as compared to group 2, (p=0.028), (Table 1, Fig. 6).

Discussion

The experimental data of our study showed that in a mice model the Ovalbumin can induce asthma, a disease in which oxidative stress and inflammation play a critical role(3). The elevated markers of lipid peroxidation (8-isoprostane level in BALF and MDA content in lung homogenate) and inflammation (total protein content and levels of IL-4 and IL-5 in BALF and IgE level in serum) support this hypothesis. There are many common assumptions concerning oxidative stress in asthma. Evidence of increased oxidative stress is the impaired endogenous antioxidant capacity in patients with asthma (3). Another important observation is that treatment with antioxidants reduces airway inflammation and hyperreactivity in asthma (15,16). We used a very small dose of MnTnHex-2-Pyp, (0.1 mg/kg/day), divided into two portions. According to Pollard et al., the D50 (toxic dose 50%) value of MnTnHex-2-PyP for subcutaneous administration was 12.5 mg (17).

Figure 4. The level of IgE in serum. (Each point represents the mean±SD for six mice.)

Source: Author

Figure 5. Level of 8-isoprostane in BALF (Each point represents the mean±SD for six mice.)

Source: Author

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MnTnHex-2-PyP\(^{5+}\) at a single dose as low as 50 \(\mu\)g/kg, which is one of the lowest, if not the lowest dose among the antioxidants commonly used in animal models of oxidative stress (18). Moreover, MnTnHex-2-PyP, a hexyl analogue of the lead compound MnTE-2-PyP, was considerably more lipophilic than MnTE-2-PyP (19) and about 30-fold more effective in protecting aerobic growth of SOD-deficient \(E.\) \(Coli\) (20). MnTnHex-2-PyP was up to 120 times more efficacious in other models of oxidative stress injury (18,19,21).

It should be noted that this antioxidant was used twice daily, before and after inhalation with OVA, because our aim was to maintain its concentration relatively constant. The parent compound, MnTE-2-PyP, in the \(i.p.\) and oral input reaches maximum concentration in the plasma for 0.33 hours. The plasma half-life at 10 mg/kg \(i.p.\) input in mice is about 1 hour and the half-life in the body ranges from 60-135 hours (22,23). As of this writing no data is available, but a study is underway exploring the MnTnHex-2-PyP pharmacokinetics (Batinić-Haberle I, unpublished data).

Metalloporphyrins have been shown to be protective in a wide range of \(in\) \(vivo\) oxidative-stress models, involving the generation of superoxide, hydrogen peroxide and peroxinitrite alone or in concert. A number of water-soluble meso-substituted manganese porphyrins with a molecular weight above 800 quickly pass through cell membranes via an unidentified transport system(s) and are distributed into the mitochondria (24). The distribution of MnTnHex-2-PyP in the liver cells are observed to be localized mainly in the mitochondria (about 90%). Mitochondrial location is roughly proportional to the number of carbon atoms in the N-alkylpyridil chains and also proportional to the lipophilicity of manganese porphyrins. This explains the significant biological resolution of MnTnHex-2-PyP to suppress the oxidative stress-induced damage in different animal models (Spasojevic I, unpublished data). Introduced intratracheally, AEOL-10113 dramatically reduces airway inflammation in OVA-induced asthma mice. The antioxidant suppresses the expression of the vascular cell adhesion molecule (VCAM-1) responsible for the accumulation of inflammatory cells (25).

Figure 6: Content of malondialdehyde in lung homogenate (Each point represents the mean\(\pm\)SD for six mice.)

The effect of Mn \(meso\)-porphyrins in decreasing the inflammatory response, which manifested by lowered neutrophil infiltration may occur through various pathways, including inhibition of the expression of adhesion molecules, activation of redox-sensitive transcription factors and generation of cytokines. Such pathways would probably involve the positive loop of the oxidative injury, in other words, the levels of ROS/RNS (reactive nitrogen species), decreased by porphyrins, would modulate cellular transcriptional activity, resulting in decreased inflammatory cell and cytokine recruitment, which would in turn decrease the levels of secondary ROS/RNS.

MnTE-2-PyP, for example, drastically reduced the severity of airway inflammation as evidenced by the reduced number of eosinophils, neutrophils, and lymphocytes found in the bronchoalveolar lavage fluid. Inhibition of ovalbumin-induced airway inflammation is associated with the inhibited expression of vascular cell adhesion molecule 1 (VCAM-1), a key adhesion molecule responsible for the recruitment of inflammatory cells into the lungs of ovalbumin-challenged mice.\(^{26}\) VCAM-1 and ICAM-1 (inter-cellular adhesion molecule-1) whose gene expression is regulated by NF-kB (26), participate in the migration of eosinophils and neutrophils, and contribute to eosinophilic inflammation in animal models. After treatment with ionizing radiation the Mn porphyrins not only clean ROS/RNS, generated by the irradiation, but also inhibit excessive cell inflammation through the suppression of transcriptional activity, particularly suppressing HIF-1\(\alpha\) (hypoxia inducible factor 1\(\alpha\)) activation in a long-lasting effect (21).

The increase in markers such as MDA, the content of which was approximately twice as much as that in the control, and 8-isoprostane, the levels of which were three times higher as compared to the controls, demonstrated that OVA increased lipid peroxidation in the lungs of the asthma-induced group. According to Emelyanov et al. (27) in exhaled breath condensate...
The effects observed after the Mn porphyrins use as antioxidants resulted from mere scavenging of ROS/RNS. In addition, these compounds were also able to modulate ROS/RNS-based signaling pathways. MnTE-2-PyP, a potent SOD mimetic, showed the greatest antioxidant efficacy of Mn porphyrins in vivo. MnTnHex-2-PyP differs from MnTBAP, by its rapid reduction during inhalation on days 24, 25, 26, reduced inflammation and lipid peroxidation, measured by sensitive biomarkers in BALF, serum and lung homogenate 48 hours after the last inhalation, in the Ovalbumin-sensitized and challenged mice.

REFERENCES
19. Batinić-Haberle I, I Spasojevic, RD Stevens, et al. Manganese(III) meso tetrakis (N-ethylpyridinium-2-yl)porphyrin (MnTE-2-PyP), its hexyl analogue MnTnHex-2-Pyp, and Mn(III) meso-tetrakis(N,N-diethylimidazolium-2-yl)porphyrin carry positively charged ortho-pyridyl or di-ortho imidazolyl moieties near the metal site (35). Another critical parameter for the in vivo efficacy of Mn porphyrins is their bioavailability. MnTnHex-2-PyP differs from MnTBAP, by its rapid reduction in vivo by numerous flavoenzymes (36,37). This quick reduction plays a major role in the antioxidant activity of this and other Mn (III) ortho-N-alkylporphyrin (37).

The effects observed after the Mn porphyrins use as antioxidants resulted from mere scavenging of ROS/RNS. In addition, these compounds were also able to modulate ROS/RNS-based signaling pathways. MnTE-2-PyP, a potent SOD mimic/ONOO⁻ scavenger, can strongly inhibit excessive activation of redox-sensitive cellular transcriptional activity.

Conclusion
The results from our experimental study revealed that MnTnHex-2-PyP, administered i.p twice daily at a dose of 0.05mg/kg during inhalation on days 24, 25, 26, reduced inflammation and lipid peroxidation, measured by sensitive biomarkers in BALF, serum and lung homogenate 48 hours after the last inhalation, in the Ovalbumin-sensitized and challenged mice.
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