Acetylcholine beyond bronchoconstriction: a regulator of inflammation and remodeling
Kistemaker, Loes

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CHAPTER 5

ANTI-INFLAMMATORY EFFECTS OF ACETYLCOLINE INHIBITION AFTER TARGETED LUNG DENERVATION IN PATIENTS WITH COPD

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Abstract

Rationale
Acetylcholine does not only induce bronchoconstriction, but is also identified as a regulator of airway inflammation in animal models of COPD. This has not been demonstrated in patients with COPD. Therefore, the effect of targeted lung denervation (TLD) on inflammation in patients with COPD was investigated in this study. TLD is a novel bronchoscopic therapy for COPD, in which airway nerves are ablated by locally applying radiofrequency energy.

Methods
Markers of inflammation in bronchial washings and brushes were investigated 30 days after TLD.

Results
TLD attenuated airway inflammation, with a significant decrease in CCL4 (MIP-1β) protein levels in the bronchial wash and CXCL8 and TGF-β gene expression levels in the bronchial brush.

Conclusions
These findings suggest that parasympathetic denervation reduces inflammation in patients with COPD.

To the editor
Acetylcholine is the primary parasympathetic neurotransmitter in the airways, and induces bronchoconstriction. Animal models of COPD revealed that acetylcholine also promotes airway inflammation, which can be inhibited by anticholinergic intervention (chapter 2). This could be clinically relevant, but has never been demonstrated in COPD patients.

We investigated the effect of targeted lung denervation (TLD) on airway inflammation in COPD. TLD is a novel potential therapy for COPD, in which parasympathetic airway nerves are bronchoscopically ablated by applying radiofrequency energy. Clinical evidence in COPD suggests that TLD improves lung function and quality of life (1). We hypothesized that TLD would inhibit airway inflammation.

Seven patients with moderate to severe COPD were recruited as part of a safety and technical feasibility study for TLD (NCT01483534) (1). TLD of the right lung was performed on day 0 (see supplement). A bronchial wash and brush were collected from the lung distal to the site of denervation before (day 0) and after denervation (day 30). We
analyzed inflammatory cells and cytokines in the bronchial wash, and gene expression of inflammatory markers in the brush.

Patient characteristics are presented in Table 1, the main results in Figure 1, and all inflammatory cells and cytokines in Table S1. The percentage of neutrophils in the bronchial wash was decreased after TLD in 5/7 patients, CXCL8 decreased in 4/7 patients, and CCL4 decreased in 6/7 patients (p=0.047). Gene expression of CXCL8 in the brush decreased in 6/7 patients (p=0.031), as did IL-6 in 5/7 patients, TGF-β in 6/7 patients (p=0.047), and MUC5AC in 5/7 patients.

Our findings suggest that TLD attenuates airway inflammation. Evidence on the role of acetylcholine in inflammation from patients is limited. Although the use of tiotropium bromide is associated with a reduction in exacerbation frequency (2), methodological problems have hampered the evaluation of inflammation in the available drug study (3). This bronchoscopic targeting of the parasympathetic system enabled us to examine the effects on airway inflammation in a completely novel, direct manner. Although denervation also targets sensory nerve fibers that release pro-inflammatory neuropeptides, such as substance P and calcitonin gene-related peptide, these nerves have been shown to regenerate, in contrast to cholinergic nerve fibers. We believe that these findings suggest a pro-inflammatory role for acetylcholine in the airways.

Acetylcholine is not only a neurotransmitter, but is also produced by non-neuronal cells. It has been proposed that this non-neuronal acetylcholine is responsible for the pro-inflammatory effects of acetylcholine (chapter 2). The current study suggests that neuronal acetylcholine may in fact contribute to inflammation in COPD.

**Table 1.** Baseline characteristics of subjects (n=7 subjects).

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>56.4 (10.4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male (n, %)</td>
<td>2 (29%)</td>
</tr>
<tr>
<td>History of Smoking (n, %)</td>
<td>7 (100%)</td>
</tr>
<tr>
<td>Pack-Years</td>
<td>35.8 (13.7)</td>
</tr>
<tr>
<td>FEV1 (L) – Pre-bronchodilator</td>
<td>0.70 (0.13)</td>
</tr>
<tr>
<td>FVC (L) – Pre-bronchodilator</td>
<td>2.19 (0.27)</td>
</tr>
</tbody>
</table>

Data are mean (SD) unless stated otherwise. FEV1=forced expiratory volume in 1 s. FVC=forced vital capacity.
Figure 1. Percentage change at day 30 compared to day 0 in levels of neutrophils and protein expression of CXCL8 and CCL4 in bronchial wash, and in gene expression of CXCL8, IL-6, TGF-β and MUC5AC in bronchial brush. Baseline values are set to 100%, represented by the dashed line. * p<0.05 (Wilcoxon signed rank test). CXCL8: C-X-C chemokine 8, also known as IL-8; CCL4: chemokine C-C ligand 4, also known as MIP-1β; IL-6: interleukin-6; TGF-β: transforming growth factor-β. In this small study population, a repeated measures analysis of covariance did not show significant interactions of these parameters with changes in FEV₁ in response to TLD.

Furthermore, TLD might also have effects on airway remodeling, since a significant reduction in TGF-β was observed. This aligns well with the reduction in TGF-β we have previously found in muscarinic M₃ receptor knock-out mice (chapter 3), and with the human data from the UPLIFT trial, where tiotropium reduced lung function decline in a subgroup of patients (4).

There are limitations to this small cohort pilot study, including the limited number of subjects and the lack of control of successful denervation. However, we believe that the findings are promising, and a large-scale multicenter sham-controlled study of the effectiveness of TLD is planned and will include similar analyses. This should provide more evidence for the role of neuronal acetylcholine as a pro-inflammatory mediator. Moreover, it would be interesting to perform similar analyses in patients treated with anticholinergic drugs.

In conclusion, our findings constitute exploratory evidence for a role of acetylcholine in airway inflammation in patients with COPD. This is a novel and sparsely explored mechanism that could be relevant for treatment strategies, but first asks for confirmation.
Acknowledgements

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Supplement

Methods

Details of TLD Procedure
Targeted lung denervation (TLD) was performed using a bronchoscopically guided catheter based lung denervation system (Holaira, Inc., Plymouth, MN, USA) delivered during two rigid bronchoscopic procedures 30 days apart, performed under general anesthesia in an outpatient setting. The lung denervation system includes a catheter with a stainless steel internally cooled electrode, thermocouple, expandable balloon, and coolant entry/exit tubing that are attached to a separate radiofrequency generator, cooling console, and coolant pump. The cooled electrode is designed to generate therapeutic lesions at a sufficient depth from the inner surface of the main right and left bronchus to ablate the airway nerves that travel parallel to and outside of the main bronchi and into the lungs. The expandable balloon provided protective cooling to minimize airway wall effects of the main bronchi during radiofrequency ablation of the nerves. The electrode was placed and activated as prescribed in up to 8 positions per bronchus to ensure complete circumferential treatment. Bronchoscopic and fluoroscopic visualization was used to guide electrode positioning.

Analysis on bronchial wash and brush
On the bronchial wash, a differential cell count was performed, and cytokine concentrations were measured. For a differential cell count, cytospin-preparations were stained with May–Grünewald and Giemsa (both Sigma, St. Louis) and a cell count was performed by counting at least 400 cells in duplicate in a blinded fashion. Levels of 26 cytokines in the wash were determined using a multiplex assay (Millipore, Billerica, MA, USA). The following cytokines were assessed: eotaxin, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, and TNF-β. From the bronchial brush, total RNA was extracted (Qiagen, Venlo, The Netherlands), reverse transcribed, and subjected to qRT-PCR. qRT-PCR was performed with denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds for 40 cycles followed by 10 minutes at 72°C. Real-time PCR data were analyzed using the comparative cycle threshold (Ct: amplification cycle number) method. The amount of target gene was normalized to the endogenous reference gene 18S ribosomal RNA.
Table S1. Levels of inflammatory cells and cytokines in bronchial wash fluid. CCL2: also known as MCP-1; CCL4: also known as MIP-1β; CCL11: also known as eotaxin; CXCL8: also known as IL-8; CXCL10: also known as IP-10. Other cytokines were below the detection limit of 3.2 pg/ml.

<table>
<thead>
<tr>
<th>Cells in wash (% of total cells)</th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
<th>Subject 5</th>
<th>Subject 6</th>
<th>Subject 7</th>
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<tr>
<td>Neutrophils</td>
<td>32.45</td>
<td>17.19</td>
<td>18.40</td>
<td>20.13</td>
<td>22.48</td>
<td>19.19</td>
<td>60.21</td>
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<td>Macrophages</td>
<td>66.30</td>
<td>81.63</td>
<td>80.66</td>
<td>73.83</td>
<td>78.88</td>
<td>38.35</td>
<td>58.38</td>
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<tr>
<td>Macrophages</td>
<td>1.06</td>
<td>1.19</td>
<td>0.69</td>
<td>0.94</td>
<td>2.06</td>
<td>1.75</td>
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<td>Lymphocytes</td>
<td>0.19</td>
<td>0.00</td>
<td>0.25</td>
<td>0.00</td>
<td>1.62</td>
<td>0.19</td>
<td>1.19</td>
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<td>Eosinophils</td>
<td>12.46</td>
<td>5.19</td>
<td>12.71</td>
<td>4.73</td>
<td>5.20</td>
<td>4.41</td>
<td>7.83</td>
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<tr>
<td>Proteins in wash (pg/ml)</td>
<td>32.32</td>
<td>5.80</td>
<td>10.71</td>
<td>0.00</td>
<td>12.12</td>
<td>0.00</td>
<td>12.65</td>
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<td>CCL2</td>
<td>32.85</td>
<td>98.26</td>
<td>303.31</td>
<td>98.65</td>
<td>146.73</td>
<td>89.49</td>
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<td>CCL4</td>
<td>231.51</td>
<td>80.04</td>
<td>195.21</td>
<td>42.55</td>
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<td>65.51</td>
<td>145.77</td>
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<td>CCL11</td>
<td>108.24</td>
<td>39.21</td>
<td>94.48</td>
<td>38.91</td>
<td>33.00</td>
<td>42.11</td>
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<td>CCL8</td>
<td>19.42</td>
<td>5.59</td>
<td>21.18</td>
<td>4.81</td>
<td>4.67</td>
<td>3.91</td>
<td>21.49</td>
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<tr>
<td>CXCL10</td>
<td>11.70</td>
<td>4.00</td>
<td>5.18</td>
<td>1.19</td>
<td>2.74</td>
<td>2.74</td>
<td>4.00</td>
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<tr>
<td>IFN-α2</td>
<td>10.04</td>
<td>7.34</td>
<td>9.43</td>
<td></td>
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<td>IL-6</td>
<td>107.00</td>
<td>30.00</td>
<td>10.00</td>
<td>3.00</td>
<td>5.00</td>
<td>1.00</td>
<td>2.00</td>
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<tr>
<td>IL-7</td>
<td>106.00</td>
<td>30.00</td>
<td>10.00</td>
<td>3.00</td>
<td>5.00</td>
<td>1.00</td>
<td>2.00</td>
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Lung denervation attenuates inflammation.
References


