The effect of inhaled thiorphan on allergen-induced airway responses in asthmatic subjects

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Summary

Background Neuropeptides are likely to be implicated in the pathophysiology of allergen-induced airway responses. However, upon release in the airways, neuropeptides are potentially inactivated by neutral endopeptidase (NEP).

Objective We hypothesized that NEP-inhibition by inhaled thiorphan (TH) would increase allergen-induced early (EAR) and late (LAR) asthmatic responses, and allergen-induced airway hyperresponsiveness to histamine in asthmatic subjects in vivo. The dose and dosing intervals of TH were derived from previous pharmacokinetic and dose-finding studies.

Methods Nine non-smoking, atopic, asthmatic men with dual asthmatic responses to inhaled house-dust mite extract participated in a double-blind, placebo-controlled, cross-over study. During each study period PC_{20} histamine was measured 24 h before, and 3 and 24 h post-allergen. TH (1.25 mg/mL, 0.5 mL) or placebo (P) were aerosolized pre-allergen, and three times at 2 h intervals post-allergen (total dose of TH: 2.5 mg). Forced expiratory volume in one second (FEV_{1}) was recorded and expressed as percentage fall from baseline. The EAR (0–3 h) and the LAR (3–8 h) were defined as maximum percentage fall from the pre-allergen baseline and as corresponding areas under the time-response curves (AUC).

Results As compared with P, TH failed to induce an acute effect on FEV_{1} at any of the timepoints (P>0.08). There was no significant difference between P and TH in the EAR and the LAR: neither in terms of maximum percentage fall from baseline (mean±SEM: EAR: 22.3±4.7% (P) and 20.4±4.1% (TH), P=0.75; LAR: 25.2±4.7% (P) and 26.4±5.8% (TH), P=0.77) nor in terms of AUC (P=0.76). Correspondingly, the changes in PC_{20} histamine were not different between the two treatments (P>0.40).

Conclusion We conclude that four adequate doses of the inhaled NEP-inhibitor, thiorphan, failed to potentiate allergen-induced airway responses in asthma. These results suggest that either neuropeptides do not play a predominant role in allergen-induced airway responses, or that allergen challenge induces NEP-dysfunction in humans in vivo.

Keywords: asthma, bronchial provocation tests, membrane metallo-endopeptidase, thiorphan

pathophysiology and maintenance of asthma [1–3]. Upon stimulation of the sensory nerves, several neurotransmitters are released in the airways conveying various proinflammatory effects through activation of specific neurokinin receptors [1,2]. SP and NKA possess bronchoconstrictor (e-NANC) activity in asthmatic [4–7] and non-asthmatic humans in vivo [4,8]. Supplementary to the acute physiological effects, these neuropeptides may also possess immunoregulatory [9–11] and proinflammatory [1–3] activities, often referred to as 'neurogenic inflammation'.

The involvement of neuropeptides in atopie asthma and allergen challenge is supported by several human and animal studies. In an autopsy study, an increased number and length of SP-immunoreactive nerve fibres has been found in bronchial tissue of asthmatics as compared with non-asthmatics [12]. Furthermore, in atopic asthmatic patients, elevated levels of SP have been measured in bronchoalveolar lavage at baseline, with further increase after a segmental allergen challenge [13]. In guinea-pigs, neuropeptide depletion by capsaicin pretreatment in vivo prevented ovalbumin-induced tracheal hyperreactivity in vitro [14], and likewise, the combination of a potent NK1- and NK2-receptor antagonist attenuated allergen-induced bronchoconstriction in vivo [15].

Neuropeptides are commonly degraded and inactivated by proteases, particularly by neutral endopeptidase (NEP; EC 3.4.24.11) [16]. NEP is a membrane-bound metalloproteinase which is present within the airways, and which preferentially cleaves neuropeptides at the amino-site of hydrophobic residues [17]. Hence, preventing the breakdown of neuropeptides by NEP-inhibitors might be a useful tool for examining the role of both exogenous and endogenous neuropeptides in asthma. Indeed, in previous human studies, inhaled NEP-inhibitors, such as thiorphan and phosphoramidon, have been shown to potentiate the bronchoconstrictor response not only to exogenous NKA in non-asthmatic [8] and asthmatic subjects [6], but also to inhaled leukotriene D4 [18] and metabisulfite [19] in non-asthmatic, and to bradykinin in asthmatic individuals [20].

Based on these data, we hypothesized that NEP-inhibition by inhaled thiorphan would amplify the contribution of endogenously released bronchoconstrictor neuropeptides during allergen-induced airway responses in asthmatic subjects in vivo. Therefore, we tested the effect of repeated doses of inhaled thiorphan on the early and the late asthmatic responses to allergen, as well as on allergen-induced airway hyperresponsiveness to histamine at 3 and 24 h post-allergen in mild to moderately asthmatic subjects.

### Materials and methods

#### Subjects

Nine non-smoking men with mild to moderate and stable asthma participated in the study (Table 1). All subjects had a history of episodic chest tightness and wheezing, especially following exposure to house dust mite but denied any other relevant disease. None of them had had any allergen exposure or respiratory tract infection for at least 4 weeks before and during the study, and none was taking concomitant medication, except for on

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* Atopic status as determined by the number of weal responses to 16 common allergen extracts (Vivodiagnost, ALK, Benelux).
§ Baselines in percentage of the predicted values in the screening period.
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demand inhaled short-acting \( \beta_2 \)-agonists. Atopy was determined by positive weal (\( \geq 3 \) mm) responses to house dust mite extract from a standardized skin-prick test kit containing 16 common airborne allergen extracts (Vivodiagnost, ALK, Benelux). Their baseline forced expiratory volume in one second (FEV\(_1\)) was \( \geq 78\% \) of predicted [21]. All subjects were hyperresponsive to inhaled histamine (PC\(_{20} \leq 4.0 \) mg/mL). Before testing, caffeine containing beverages and the \( \beta_2 \)-agonists were withheld for at least 4 and 8 h, respectively. The study was approved by the Ethics Committee of the Leiden University Hospital and all participants gave their informed consent.

**Study design**

Before entering the study, all subjects were seen within 1 week on three separate screening visits during which the selection criteria were examined. On screening day 1, a questionnaire, a PC\(_{20} \) histamine and a skin-prick test were performed. On screening day 2, subjects underwent an allergen diluent challenge and a multi-dose skin-prick test with house dust mite extract for the determination of the skin-test sensitivity [22]. Based on the data of the previous two visits, a standardized allergen challenge test with inhaled house dust mite was performed on screening day 3 [22,23]. Subjects were enrolled in the study provided that they had a documented EAR (fall in FEV\(_1\) \( \geq 15\% \)) from baseline, between 3 and 7 h post-allergen and LAR (defined for inclusion as a fall in FEV\(_1\) \( \geq 15\% \)) from baseline) for 7 h after inhalation of the diluent of allergen [23]. Furthermore, prestudy physical examination and ECG had to be within normal limits. The study had a randomized, double-blind, placebo-controlled, and cross-over design, and consisted of two periods of 3 days each, separated by a washout interval of at least 14, and maximally 42 days. Tests were performed at the same time of the day (\( \pm 2\) h) in each individual. Baseline FEV\(_1\) on days 1 and 2 of both study periods had to be within 10%. Furthermore, PC\(_{20} \) histamine on day 1 of both study periods had to remain within one doubling concentration. On day 2 of both study periods, subjects inhaled either thiorphan or placebo, before and repeatedly at 2 h intervals after a standardized allergen challenge. Furthermore, a histamine challenge test was performed at 3 h post-allergen and the PC\(_{20} \) histamine was calculated. On study day 3, 24 h post-challenge, another PC\(_{20} \) histamine was recorded.

**Inhalation challenges**

Histamine challenges were performed using histamine-acid-phosphate (Sigma Chemicals, St Louis, MO, USA). Solutions of histamine were stored at 4°C until 30 min before the tests and administered at room temperature. The tests were performed according to a standardized method [23]. Briefly, doubling concentrations of histamine (0.03–4 mg/mL in phosphate-buffered saline [PBS]) were aerosolized by a DeVilbiss 646 nebulizer (output 0.13 mL/min) and inhaled by tidal breathing through the mouth with the nose clipped, during 2 min at 5 min intervals until a \( \geq 20\% \) fall from baseline was reached.

Allergen inhalation challenge tests were also performed according to a standardized protocol [22,23]. Purified aqueous allergen extract of *Dermatophagoides pteronyssinus* (SQ 503, Vivodiagnost, ALK, Benelux) was prepared from the same production batch for both the multi-dose skin-prick tests and inhalation challenge tests and stored at 4°C. Before the study, the extract was diluted in Vivodiagnost diluent (PBS with 0.03% human serum albumin and 0.5% phenol as a preservative). Each subject had his own stock solution of 5 mL, containing 10,000 BU/mL of allergen extract, which was diluted to 25 mL and divided in five equal portions of 5 mL, containing 2000 BU/mL each. Immediately before challenge, doubling dilutions were prepared from the stock solution ranging from 2000–15.63 BU/mL in 3 mL diluent. PC\(_{20} \) allergen was predicted from PC\(_{20} \) histamine and the skin-test sensitivity, derived from the multi-dose skin-prick test, according to Cockcroft's method [22]. Starting two dilutions below the predicted PC\(_{20} \) allergen, consecutive doubling dilutions of allergen were aerosolized at 10 min intervals until a drop in FEV\(_1\) of \( \geq 20\% \) occurred, using the same method and equipment as for the histamine inhalations. During study periods I and II, each subject inhaled the same three dilutions of house dust mite extract, being the three highest ones which had evoked both an early and a late asthmatic response during the screening period.

Thiorphan (DL-3-mercapto-2-benzylpropanoylglutamylcine; Sigma Chemicals, St Louis, MO, USA, in normal saline containing 1% human serum albumin; CLB, Amsterdam, The Netherlands) was administered using a validated method [6,8,18]. For the determination of the dose and dosing intervals of inhaled thiorphan, we considered the following. In previous dose-finding experiments, pretreatment with inhaled thiorphan in a single dose of 0.62 mg or 1.25 mg has been shown to produce similar, i.e. maximal potentiation of the bronchoconstrictor response to exogenous NKA in asthmatic subjects [6]. Furthermore, without affecting the airway response to methacholine in asthmatics [6], 1.25 mg of inhaled thiorphan significantly enhanced the maximal response plateau to other pro-inflammatory stimuli, such as inhaled LTD\(_4\) [18] and metabisulphite [19] in non-asthmatic...
humans. Although in a pharmacokinetic study in guinea-pigs, inhaled thiorphan has been shown to possess a short duration of action (± 5 min) in potentiating the effect to inhaled SP [24], in previous studies in non-asthmatic subjects, we have shown that 1.25 mg of inhaled thiorphan has enhanced the maximum airway narrowing to inhaled NKA or LTD₄ for at least 1½ h [8,18]. Based on these data and a safety pilot study, we determined the dosing intervals at 2 h, using inhaled thiorphan in a dose of 0.62 mg at each timepoint. To this aim, we administered four doses, each consisting of 0.5 mL of a 1.25 mg/mL of a thiorphan solution, aerosolized immediately before, and 2, 4, and 6 h following allergen inhalation, yielding a cumulative dose of 2.5 mg thiorphan. Thiorphan was stored at −20 C and warmed up on melting ice before nebulization. The aerosols were generated using a highly efficient jet-nebulizer (Mallinckrodt Diagnostics, The Netherlands) [6,8,18]. This nebulizer was filled with 0.5 mL of each concentration, which was sprayed during 1 min by compressed nitrogen into a 30 L collapsible drying chamber, in which the droplets (saline mass median aerodynamic diameter (MMAD) 2.5 μm) quickly evaporate to dry particles (MMAD 0.6 μm). Following nebulization, the aerosols were inhaled by tidal breathing with the nose clipped, through a three-way valve box and a mouthpiece for 3–4 min/dose. Oxygen was supplied into the mouthpiece (4 L/min).

Response measurements

The airway response to the inhaled aerosols was recorded according to standard lung function techniques [21], using FEV₁ obtained from complete expiratory flow-volume curves, which were recorded on a dry-rolling-seal spirometer (Morgan Spirosflow, PK Morgan, UK) in series with a plotting system (Kipp, BD 90, Delft, The Netherlands). Before each test, FEV₁ was measured in triplicate for the calculation of the mean baseline value [23]. The response to histamine was obtained by an FEV₁ measurement at 45s after each dose until a ≥ 20% fall from baseline was reached. The provocative concentration producing a 20% fall from baseline FEV₁ (PC₂₀) was calculated by interpolation [23]. After the histamine challenges, subjects received inhaled salbutamol as needed, except for those challenges performed at 3 h post-allergen. The airway response to thiorphan and placebo treatment was measured by FEV₁ in triplicate immediately after each dose. Pre-allergen baseline, being the average of the FEV₁ values after the first treatment dose, was used in the analysis of the allergen challenges.

The airway response to allergen was measured in duplicate 10 min after each inhalation of allergen, and was repeated at 10, 20, 30, 40, 50, 60 min and then hourly up to 8 h after the last inhalation [23]. At each timepoint, the largest FEV₁ was applied for analysis. After allergen challenge, all subjects received inhaled salbutamol and, provided the FEV₁ had returned within 20% of pre-allergen baseline (or an FEV₁ > 3L), they were sent home with written instructions, salbutamol and a portable spirometer. In addition they were asked not to use any salbutamol for 8 h before testing on the next day.

Analysis

Histamine concentration–response curves were characterized by their position [23], expressed as PC₂₀. Natural log transformations were applied to the PC-values before statistical analysis.

The airway response to inhaled allergen was expressed as percentage fall from preallergen baseline and plotted against the time in minutes or hours (time–response curves). In the analysis, the EAR was defined as the maximum percentage fall from baseline FEV₁ and as the area under the time–response curve (AUC) from 0 to 3 h post-allergen. Likewise, the LAR was defined as the maximum percentage fall in FEV₁ and as the AUC from 3 to 8 h post-allergen [23]. The corresponding AUCs were calculated by trapezoidal rule.

Student’s paired two-tailed t-tests were applied to test the differences in EAR, LAR, and ΔPC₂₀ between thiorphan and placebo treatment. P-values less than 5% were considered statistically significant. Based on recent data [25], a sample size of nine subjects was considered to be sufficient to detect a mean change of 35% in the EAR and of 30% in the LAR (power: 0.9; a = 0.05, two-tailed; β = 0.10, one-tailed).

Results

Safety

Thiorphan, allergen extract, and the combination of both were well-tolerated in all subjects and no adverse reactions were noted. All subjects completed the study and none of them needed rescue medication before 8 h post-allergen. Likewise, none of the patients needed additional salbutamol within 8 h before the tests on study day 3.

Airway calibre

Although there was a slight but significant difference between the pre-treatment baselines, i.e. before administering thiorphan or placebo (mean difference ± SEM: 0.11 ± 0.04 L; p = 0.03), there were no significant differences in the changes in baseline FEV₁ between the two...
The effect of inhaled thiorphan on allergen-induced airway responses in asthmatic subjects

Table 2. Baselines FEV₁ (in L) recorded immediately pretreatment, preallergen, and at 24 h postallergen during both treatment periods. Pretreatment baselines were slightly different (P = 0.03), but there was no significant difference in the changes in baseline FEV₁ between the two treatments at any of the four timepoints (P > 0.08). Preallergen baselines were not different (P = 0.53), and there was no significant difference in the changes in the baseline values at 24 h postallergen compared with pretreatment baselines, between the two treatments (P = 0.77).

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Table 2. Baselines FEV₁ (in L) recorded immediately pretreatment, preallergen, and at 24 h postallergen during both treatment periods. Pretreatment baselines were slightly different (P = 0.03), but there was no significant difference in the changes in baseline FEV₁ between the two treatments at any of the four timepoints (P > 0.08). Preallergen baselines were not different (P = 0.53), and there was no significant difference in the changes in the baseline values at 24 h postallergen compared with pretreatment baselines, between the two treatments (P = 0.77).

Airway hyperresponsiveness

At 3 h post-allergen there was a fall in PC₂₀ histamine during placebo which failed to reach significance (mean

![Fig. 1. Airway responses to inhaled house-dust mite extract (expressed as percentage change from baseline FEV₁; mean ± SEM) between 0 and 8 h following allergen challenge during placebo (○) and thiorphan (●) treatment. Neither the EAR (0–3 h) nor the LAR (0–8 h) were significantly enhanced by thiorphan as compared to placebo (> 0.75).](image-url)
Fig. 2. Airway responses to inhaled house-dust mite extract (expressed mean as AUC ± SEM) during the EAR and the LAR. During the EAR and the LAR, there was no significant difference in AUC between the two treatments (P > 0.76).

\[ \Delta \text{PC}_{20} \pm \text{SD} = 0.72 \pm 1.11 \text{ doubling concentrations, } P = 0.09 \]. In contrast, a significant fall in PC\(_{20}\) histamine at 3 h post-allergen occurred after thiorphan treatment (mean \( \Delta \text{PC}_{20} \pm \text{SD} = 1.05 \pm 0.89 \text{ doubling concentrations, } P = 0.008 \)). However, these changes in PC\(_{20}\) were not significantly different between the two treatments (P = 0.40) (Table 3).

Allergen-induced airway hyperresponsiveness to histamine at 24 h post-allergen was significantly enhanced following both placebo and thiorphan treatment (mean \( \Delta \text{PC}_{20} \pm \text{SD} = 1.18 \pm 1.29 \text{ doubling concentrations, } P = 0.03 \), and 1.13 \pm 0.73 doubling concentrations, P = 0.002, respectively). However, these changes in PC\(_{20}\) were not significantly different between the two treatments (P = 0.90) (Table 3).

Discussion

The findings of the present study indicate that NEP-inhibition by repeated doses of inhaled thiorphan does neither increase allergen-induced airway responses during the early and late asthmatic reaction, nor the allergen-induced airway hyperresponsive to histamine at 3 and 24 h post-allergen in asthmatics in vivo. This suggests that, in humans, either secondary release of endogenous bronchoconstrictor neuropeptides does not play a predominant role in allergen-induced airway responses, or that allergen-challenge induces NEP-dysfunction within the airways.

This is the first intervention study in humans aimed at elaborating the pathophysiological role of endogenous neuropeptides and NEP-activity during allergen challenge in vivo. In guinea-pig studies, various tools have been applied to investigate the role of endogenous neuropeptides in allergen-induced airway responses. Depletion of neuropeptides by pretreatment with capsaicin before or after sensitization with OVA, prevented both the OVA-induced bronchospasm [26], and the OVA-induced airway hyperresponsiveness in vivo [27]. Likewise, the NEP-inhibitors thiorphan and phosphoramidon potentiated allergen-induced bronchoconstriction in guinea-pigs both in vitro and in vivo [28,29]. Correspondingly, in two in vivo guinea-pig studies, a selective NK\(_1\)-receptor antagonist attenuated allergen-induced plasma extravasation in the airways [30], and in combination with a potent NK\(_2\)-receptor antagonist, abolished the allergen-induced bronchoconstriction [15]. Although from these studies it can be concluded that neuropeptides do contribute to allergen-induced airway responses, we could not confirm this hypothesis using NEP-inhibition by inhaled thiorphan in asthmatic humans in vivo.

We do not believe that our negative results can be explained by methodological factors. Firstly, we have selected a group of volunteers with similar characteristics as the subjects in whom Cheung et al. [6] previously

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| Table 3. PC\(_{20}\) histamine (in mg/mL) ± sd (in doubling concentrations) at 24 h before, and 3 and 24 h postallergen during both treatment periods. There was no significant difference in the changes in PC\(_{20}\) histamine at any of the timepoints (P > 0.40).
showed evidence of endogenous NEP-activity in vivo by the potentiating effect of inhaled thiorphan on inhaled neuropeptide A. Factors potentially causing impaired NEP-function, such as smoking, lower airways infections and the use of corticosteroids, were excluded. Secondly, we have applied validated methods [22,23,31] which account for reproducible measurements during both study periods (Fig. 1). And finally, the present dose and dosing intervals of thiorphan administration were derived from previous observations, as was pointed out in the method section [6,8,18,19,24]. Throughout the allergen challenge, the cumulative dose of thiorphan was twice the dose which has been shown to enhance the bronchoconstrictor response to inhaled neuropeptide A in non-asthmatic and mildly asthmatic humans [6,8] and to leukotriene D$_4$ [18] and metabisulphite [19] in non-asthmatics. Since potentiation of the latter two challenges can be considered to be due to the reduced breakdown of endogenous neuropeptides, it seems likely that the present dose of thiorphan would have been sufficient to inhibit the cleavage of neuropeptides that are released secondary to allergen challenge.

How can we explain the lack of effect of thiorphan on allergen-induced airway responses in asthma? Theoretically, inhalation of thiorphan might result in a balanced potentiation of i-NANC and e-NANC responses after allergen challenge [16,17], leaving the EAR and the LAR unchanged. However, previous animal studies do not favour a role of i-NANC in allergen-induced airway responses [28,29], and if anything, have provided evidence for a dysfunction of i-NANC after allergen challenge [32]. Another explanation of our findings can be a dysfunction of either e-NANC or NEP following allergen challenge. Indeed, two studies have demonstrated that a high dose of capsaicin given subcutaneously after ovalbumin challenge, did neither affect airway microvascular permeability [33] nor airway resistance [34] in OVA-sensitized guinea-pigs. This is suggestive of an impaired release of endogenous neuropeptides following allergen challenge, since exaggerated bronchoconstrictor responses to exogenous neuropeptides (SP and NKA) could still be demonstrated in sensitized guinea-pigs after allergen inhalation, whereas the responses to dry gas hyperpnoea and i.v. capsaicin remained unchanged [35]. Interestingly, Lilly et al. [36] provided recent evidence of impaired NEP-activity in guinea-pig lungs after allergen challenge. They showed that the selective NEP-inhibitor SCH 32,615 failed to potentiate the bronchoconstrictor response to exogenous SP in guinea-pig airways after chronic allergen exposure, whereas it did after saline [36]. In addition, the observation that allergen-induced hyperresponsiveness to inhaled bradykinin is much more pronounced than that to methacholine in asthmatic subjects in vivo [37], fits in with the hypothesis that allergen challenge may lead to NEP-impairment, as has also been observed with other inflammatory stimuli, e.g. viral infections [38], cigarette smoke [39], and toluene diisocyanate [40]. Therefore, it may not be surprising that treatment with thiorphan failed to potentiate allergen-induced airway responses in subjects with atopic asthma in the present study.

In conclusion, the results of the present study indicate that either neuropeptides do not play a predominant role in the allergen-induced airway responses, or that allergen challenge induces NEP-dysfunction in humans. With the tools used in the present study, we were not able to differentiate between these two possibilities. Hence, evaluation of the exact role of neuropeptides in allergen-induced airway responses awaits the availability of potent and selective neuropeptide receptor antagonists for human use. In addition, the potential impairment of NEP-activity following allergen challenge needs further examination by, e.g. immunohistochemical techniques.

References


