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Effect of an inhaled neutral endopeptidase inhibitor, thiorphan, on airway responsiveness to leukotriene D_4 in normal and asthmatic subjects

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ABSTRACT: Cysteinyl leukotrienes are potent inflammatory mediators that are considered to play a role in the pathophysiology of asthma. It can be postulated that leukotrienes exert their bronchoconstricting effects, in part, through secondary release of endogenous neuropeptides.

We examined the effect of inhaled thiorphan, an inhibitor of a neuropeptide degrading enzyme, on the concentration-response curve to leukotriene D_4 ($LTD_{4)}$ in a two-period, double-blind, cross-over and placebo-controlled study, in 16 nonasthmatic and 12 asthmatic subjects. Thiorphan or placebo were aerosolized and administered in two 0.5 ml doses of 1.25 mg·ml^{-1} each, 10 min prior to LTD_4 inhalation. The airway response was measured by forced expiratory volume in one second (FEV_1) and partial expiratory flow-volume curves (expiratory flow at 40% of forced vital capacity; $\dot{V}_{40p}$), and expressed as % fall from baseline. Complete concentration-response curves to inhaled LTD_4 were recorded and characterized by their position (provocative concentration producing a 20% fall in FEV_1 and a 40% fall in $\dot{V}_{40p}$; $PC_{20}FEV_1$ and $PC_{40}\dot{V}_{40p}$) and, in the nonasthmatics, also by the maximal-response plateau ($MFEV_1$,$M\dot{V}_{40p}$).

Post-pretreatment baseline values of FEV_1 and $\dot{V}_{40p}$ were not different between thiorphan and placebo pretreatment. In both groups of subjects, there was no significant difference in $lnPC_{40}\dot{V}_{40p}$ or $lnPC_{20}FEV_1$ to LTD_4 between the two pretreatments mean difference±SD (in doubling concentrations): 0.12±0.73 and -0.19±1.23, respectively, in asthmatics; and 0.17±0.95 and -0.99±1.95, respectively, in nonasthmatics. The maximal-response plateau could not be obtained in the majority of the asthmatic subjects. In the normals, however, $M\dot{V}_{40p}$ was significantly increased by thiorphan as compared to placebo pretreatment (mean difference±SD: 5.7±8.1 % fall; p=0.013) whereas the difference in $MFEV_1$ failed to reach significance (mean difference±SD: 2.7±5.3% fall).

We conclude that thiorphan slightly potentiates maximal airway narrowing to inhaled LTD_4 in normal humans *in vivo*, without affecting the sensitivity to LTD_4. These findings suggest that cysteinyl leukotrienes exert their inflammatory effects, at least in part, by the release of endogenous neuropeptides in human airways *in vivo*.

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Atopic asthma is a chronic inflammatory disorder of the airways associated with airway hyperresponsiveness to various bronchoconstrictor stimuli [1]. Although there is increasing evidence for a relationship between airway inflammation and hyperresponsiveness, the mechanisms by which inflammatory cells, their products, and bronchial nerves interact to produce asthmatic responses are still uncertain [1, 2].

Cysteinyl leukotrienes (LTC_4, LTD_4 and LTE_4) are metabolites of arachidonic acid formed by the 5-lipoxygenase pathway, representing a heterogeneous group of biologically active mediators [3]. The bronchoconstricting properties of these mediators include contraction of airway smooth muscle, increase in microvascular permeability, mucus hypersecretion, chemotaxis and activation of inflammatory cells, which by themselves can release secondary mediators [3, 4]. Several investigators have shown that leukotrienes induce airway narrowing [4, 5], and even hyperresponsiveness to histamine or methacholine [6, 7] in both normal and asthmatic subjects. Moreover, during acute exacerbations of asthma, the presence of leukotrienes has been demonstrated in sputum, bronchial lavage fluid, and plasma [8], and enhanced urinary LTE_4 levels have been found in sensitized subjects following allergen challenge [9]. In addition, pretreatment with specific leukotriene receptor

antagonists attenuates early and late asthmatic responses [10, 11], and allergen-induced airway hyperresponsiveness [11]. Consequently, it has been postulated that leukotrienes play a role in the pathophysiology of asthma.

Apart from their bronchoconstricting effects by stimulation of specific receptors on *e.g.* airway smooth muscle or endothelial cells [12], there is increasing evidence from animal studies that leukotrienes exert their inflammatory effects partly through secondary release of endogenous neuropeptides, such as substance P and neurokinin A [13–17]. These neuropeptides are released by stimulation of sensory C-fibre nerve endings in the bronchial wall, also called nonadrenergic noncholinergic (NANC) or capsaicin-sensitive nerves [18–20]. These potent bronchoconstricting substances have been shown to increase smooth muscle tone, vascular permeability, cholinergic neurotransmission, active epithelial chloride transport and to induce coughing [18–20]. In addition, they induce and mediate several immunological processes, which may contribute to further inflammation of the asthmatic airways, leading to so-called neurogenic inflammation [18–20]. Normally, neuropeptides are inactivated by an endogenous degrading enzyme, neutral endopeptidase (NEP), which is predominantly present in airway epithelium [21]. NEP inhibitors, such as phosphoramidon and thiorphan, therefore facilitate the activity of neuropeptides [21]. This has been confirmed in both normal and mildly asthmatic humans *in vivo* by CHEUNG and co-workers [22, 23], who demonstrated that inhaled thiorphan potentiates airway narrowing to inhaled neurokinin A without affecting the airway response to methacholine. This finding implies that both normal and asthmatic subjects have endogenous NEP activity *in vivo*.

In the present study, we tested the hypothesis that the bronchoconstricting effects of inhaled LTD_4 in humans are mediated, in part, by secondary release of neuropeptides. For that purpose we examined whether pretreatment with inhaled thiorphan potentiates airway responsiveness to inhaled LTD_4 in normal and asthmatic humans *in vivo*.

Methods

Subjects

The study consisted of two parts. In part 1, 16 healthy, nonsmoking, male subjects, aged 18–41 yrs (mean 24 yrs), were recruited from volunteers in the laboratory and among students. The subjects denied any history of asthma or other relevant disease, and took no regular medication. Baseline forced expiratory volume in one second (FEV_1) was >90% of predicted [24], and airway responsiveness to methacholine was within normal limits (provocative concentration producing a 20% fall in FEV_1: PC_{20} >8 mg·ml^{-1}) [25]. All subjects were nonatopic, as was demonstrated by a negative skin-prick test to 16 common allergen extracts (wheal <3 mm; Vivodiagnost, ALK, Benelux) (table 1).

In part 2 of the study, 12 nonsmoking males with mild asthma, aged 19–31 yrs (mean 25 yrs), were enrolled.

Table 1. – Characteristics of normal subjects

Subject no.	Age yrs	Height cm	FEV_1* % pred	$PC_{20}FEV_1$** methacholine mg·ml^{-1}
1	25	190	105	17
2	26	181	107	21
3	25	192	94	27
4	21	190	97	35
5	23	198	101	38
6	21	186	115	62
7	22	177	113	71
8	40	175	92	74
9	30	180	107	122
10	18	183	118	123
11	23	180	95	195
12	29	176	109	220
13	21	190	118	447
14	21	187	121	543
15	24	182	99	>624
16	20	180	113	>624

*: the baseline value on screening day 1 in % predicted value; **: provocative concentration of methacholine bromide causing a 20% fall in FEV_1 ($PC_{20}FEV_1$) on screening day 1. FEV_1: forced expiratory volume in one second.

All had stable asthma, taking no medication, except for infrequent on demand usage of inhaled β_2-agonists. Their baseline FEV_1 was ≥70% of predicted [24], and they were all hyperresponsive to methacholine (PC_{20} <8 mg·ml^{-1}) [25]. Their atopic status was confirmed by a positive skin-prick test to at least one of the 16 common allergen extracts (wheal ≥3 mm) (table 2). The study was carried out in the autumn. Two weeks before the start of the study, all subjects were free of the symptoms of respiratory tract infection. They were asked not to use any medication during the study and to refrain from

Table 2. – Characteristics of asthmatic subjects

Subject no.	Age yrs	Height cm	atopic status†	FEV_1* % pred	$PC_{20}FEV_1$** methacholine mg·ml^{-1}
1	22	188	4	74	0.19
2	19	172	3	98	0.18
3	30	181	8	82	0.26
4	20	183	5	93	0.44
5	21	183	4	92	0.45
6	31	185	5	94	0.63
7	21	177	1	79	0.66
8	28	186	7	93	0.94
9	24	193	5	114	1.29
10	29	188	6	103	1.52
11	23	201	2	118	1.73
12	26	181	6	99	2.27

†: atopic status determined by the number of wheal responses to 16 common allergen extracts; *: the baseline value on screening day 1 in % predicted value; **: provocative concentration of methacholine bromide causing a 20% fall in FEV_1 ($PC_{20}FEV_1$) on screening day 1. FEV_1: forced expiratory volume in one second.

caffeine-containing beverages for at least 4 h before testing. The protocol was approved by the Medical Ethics Committee of the University Hospital of Leiden, and all participants gave their informed consent.

Study design

Both parts of the study had a similar design. Before entering the study, all subjects were seen on two separate screening days. On screening day 1 the inclusion criteria were examined, and on screening day 2 an LTD_4 inhalation provocation test was performed as an individual dose-searching procedure. The study consisted of two days, separated by a wash-out period of at least 14 days. On both study days, an LTD_4 inhalation test was performed, 10 min after inhalation of pretreatment consisting of either thiorphan or placebo, in a randomized, double-blind, and cross-over manner. A concentration-response curve to inhaled LTD_4 was subsequently recorded until a maximal-response plateau was reached, until the FEV_1 dropped to values <50% from baseline, or until the highest dose had been given. The variability of baseline FEV_1 was not allowed to exceed 10% between study days 1 and 2 of both study periods. Subjects attended the laboratory at the same time (±2 h) of the day. After the inhalation tests, aerosolized salbutamol was administered (200 µg per metered dose inhaler).

Inhalation challenges

The inhalation provocation tests were performed using methacholine (acetyl-β-methylcholine-bromide in normal saline; Jansen Pharmaceutica, Belgium), synthetic leukotriene D_4 dipotassium salt in phosphate-buffered saline (PBS) (Merck Frosst Laboratories, Dorval, Quebec, Canada) and thiorphan (DL-3-mercapto-2-benzylpropanoylglycine; Sigma Chemicals, St. Louis, MO, USA) in normal saline, containing 1% human serum albumin (CLB, Amsterdam, The Netherlands).

In the screening period, methacholine challenges were performed using methacholine bromide, according to the method of COCKCROFT [25]. Methacholine bromide (MeBr) was chosen because of the unavailability of methacholine chloride (MeCI) for human use (PC_{20} MeBr=1.2×PC_{20} MeCI) [26]. During the study, methacholine was stored at 4°C and administered at room temperature in doubling concentrations, ranging from 2.4–624 $mg \cdot ml^{-1}$ for the nonasthmatic and 0.14–9.6 $mg \cdot ml^{-1}$ for the asthmatic subjects, until a ≥20% fall in FEV_1 from baseline was reached. The aerosols were generated by a DeVilbiss 646 nebulizer (output 0.13 $ml \cdot min^{-1}$) connected to an inspiratory and expiratory valve box with an expiratory aerosol filter (Pall Ultipor BB50T). Each concentration was inhaled by tidal breathing through the mouth with the nose clipped, for 2 min at 5 min intervals.

Leukotriene D_4 inhalation provocation tests were also performed, according to a previously validated method [6]. Stock solutions of LTD_4 were stored at -70°C. Maximally at one hour before use, serial dilutions containing doubling concentrations (between 0.11–430 $\mu g \cdot ml^{-1}$ LTD_4 for the normal subjects and 0.0033–7.0 $\mu g \cdot ml^{-1}$ LTD_4 for the asthmatics) were prepared in 4.3 M ethanol, 0.11 M sodium chloride, and 7.5 mM phosphate (pH 7.2). All dilutions were mixed with nitrogen and kept on melting ice before nebulization. The aerosols were generated using a highly efficient jet-nebulizer (Mallinckrodt Diagnostics, The Netherlands) [6, 22]. This nebulizer was filled with 0.5 ml of each concentration, which was sprayed for 1 min by compressed nitrogen into a 30 *l* collapsible drying chamber, in which the droplets (mass median aerodynamic diameter (MMAD) 2.5 µM) evaporated to dry particles [6, 22]. Following nebulization, the aerosols were inhaled by tidal breathing, with the nose clipped, through a 3-way valve box and a mouthpiece within 3–4 min. Oxygen was supplied into the mouthpiece ($4\ l \cdot min^{-1}$). Each LTD_4 challenge was preceded by inhalation of its solvent, PBS, as a control solution. Consecutive LTD_4 concentrations were administered at ±7 min intervals.

Thiorphan was administered by a method and in a dose which have been shown to be safe and effective in humans [22, 23]. Thiorphan was stored at -20°C and warmed up on melting ice before nebulization. Prior to the beginning of the LTD_4 challenge, two consecutive 0.5 ml doses of thiorphan ($1.25\ mg \cdot ml^{-1}$) were inhaled within 10 min, using the same equipment as for LTD_4.

Airway responses were measured according to standard lung function techniques [24], using complete and partial expiratory flow-volume curves which have been standardized for volume and volume history (expiratory flow at 40% of forced vital capacity; $\dot{V}_{40p}$) [27]. Lung volume was recorded on a dry-rolling-seal spirometer (Spiroflow, Morgan/Gillingham, UK) and a plotting system (Kipp, BD 90, Delft, The Netherlands) with a timing device to provide a spike for the determination of the FEV_1. Firstly, a maximal inhalation to total lung capacity (TLC) was performed, followed by 45 s tidal breathing through the spirometer. Subsequently, a partial expiratory flow-volume curve was produced from a fixed volume (60% of the largest baseline forced vital capacity (FVC), marked off from the TLC), followed by an FVC manoeuvre for FEV_1, measurement [27]. Prior to each inhalation test, three complete and three partial expiratory flow-volume curves were performed, of which the two highest FEV_1 and $\dot{V}_{40p}$ recordings were used to calculate mean baseline values. Response measurements were performed 1.5 min after each concentration of methacholine, 1.5, 3.0 and 4.5 min after both inhalations of thiorphan, and 1.5 and 3 min after each concentration of LTD_4.

Analysis

The responses of FEV_1 and flow from the partial flow-volume curves at 40% of baseline FVC ($\dot{V}_{40p}$) were expressed as percentage fall from baseline values, and were plotted against the dose of nebulized LTD_4 in $\mu g \cdot ml^{-1}$. The LTD_4 concentration-response curves were characterized by their position and maximal-response plateau

[6, 27]. The position was expressed as the provocative concentration causing a 20% fall in FEV_1 ($PC_{20}FEV_1$) or a 40% fall in $\dot{V}_{40p}$ ($PC_{40}\dot{V}_{40p}$). A maximal-response plateau was defined as two or more highest data points on the concentration-response curve within a 5% response range [27]. The levels of the maximal-response plateaus ($MFEV_1$, $M\dot{V}_{40p}$) were calculated by averaging the consecutive points on the plateaus.

Natural log transformations were applied to the PC values before statistical analysis. Two-way analysis of variance (ANOVA) was applied to test the differences in the dose-response curves between placebo and thiorphan. This could only be applied to the normal subjects, since not all of the asthmatics had received LTD_4 in the same dose-range. Student's paired two-tailed t-test was performed to test the differences in the variables between thiorphan and placebo pretreatments. P-values less than 0.05 were considered statistically significant. The estimation of the sample size of both groups was based on paired t-test analysis [28], and on data showing that reproducibility of LTD_4 provocation tests by the present method is comparable to that of methacholine challenges [27, 29]. Knowing the SD of the difference between repeated measurements of $\ln PC_{40}\dot{V}_{40p}$ and $M\dot{V}_{40p}$ (0.58 and 7.7%, respectively) [27], the power of the analysis was 0.9 (β=0.1, one-tailed) to detect at least a 0.74 fold change in $PC_{40}\dot{V}_{40p}$, a 6.8% fall change in $M\dot{V}_{40p}$ in the normal subjects, and at least a 0.87 fold change in $PC_{40}\dot{V}_{40p}$ in the asthmatic subjects, if a=0.05 (two-tailed) [28]. These changes are sufficiently smaller than those that can be considered the smallest meaningful treatment effects on the sensitivity and maximal airway responses to the inhaled drugs in man.

Results

Effect of thiorphan versus *placebo on baseline lung function in normal subjects*

LTD_4, thiorphan and the combination of both drugs were well-tolerated in all subjects. Baseline FEV_1 was slightly higher before pretreatment with thiorphan as compared to placebo (mean difference ±SD: 0.13±0.24 (*l*); p=0.043) (table 3). Thiorphan produced a minor decrease in FEV_1 (mean difference ±SD: 0.08±0.13 (*l*), p=0.024) (table 3), whereas placebo did not (p>0.32). These changes in FEV_1 were significantly different between placebo and thiorphan (mean difference ±SD: 0.10±0.17 (*l*), p=0.036). However, post-pretreatment FEV_1 was not different between the two pretreatments (p>0.43). There were no differences in $\dot{V}_{40p}$ between placebo and thiorphan, both before and after pretreatment (p>0.14).

Effect of thiorphan versus *placebo on the concentration-response curve to LTD_4 in normal subjects*

All nonasthmatic subjects reached a maximal-response plateau to LTD_4 in both study periods (fig. 1). Two-way analysis of variance was applied on the LTD_4 dose-response curves for FEV_1 and $\dot{V}_{40p}$ after both placebo and thiorphan. Significant differences between the two pretreatments were found for different concentrations for $\dot{V}_{40p}$ only (p=0.002). When Student's paired two-tailed t-test was applied to these results, $M\dot{V}_{40p}$ increased significantly after thiorphan as compared to placebo

Table 3. – Individual values of baseline FEV_1 and $\dot{V}_{40p}$ before and after placebo or thiorphan in normal subjects

Subject	Baseline FEV_1 *l*				Baseline $\dot{V}_{40p}$ $l \cdot s^{-1}$			
	Placebo		Thiophan		Placebo		Thiorphan	
no.	Before	After	Before	After	Before	After	Before	After
1	4.75	4.75	4.94	4.98	3.13	3.15	3.55	3.50
2	4.75	4.86	5.21	4.81	7.40	7.08	6.60	6.45
3	4.56	4.64	4.64	4.52	2.65	2.80	2.45	2.45
4	4.74	4.85	5.36	5.08	4.05	4.80	5.18	4.85
5	5.09	5.04	5.17	5.13	3.60	3.58	3.68	3.95
6	5.67	5.59	5.70	5.58	5.38	5.30	6.25	5.65
7	4.52	4.55	5.02	4.91	4.23	4.53	5.88	5.28
8	3.59	3.52	3.66	3.59	2.50	2.25	2.75	2.70
9	4.60	4.60	4.48	4.37	3.15	3.20	3.00	3.05
10	5.45	5.49	5.43	5.41	6.23	6.23	6.30	5.40
11	4.25	4.21	4.10	4.16	2.88	3.00	2.75	2.80
12	4.64	4.55	4.51	4.44	4.30	4.35	4.10	3.73
13	5.56	5.67	5.86	5.78	5.35	5.30	7.25	6.75
14	5.56	5.55	5.64	5.74	4.20	4.55	3.95	4.35
15	4.66	4.77	4.87	4.89	2.90	3.18	3.30	3.58
16	5.25	5.29	5.14	5.12	5.55	5.55	5.10	5.45
Mean	4.85	4.87	4.98	4.91	4.22	4.30	4.51	4.37
SD	0.55	0.57	0.60	0.59	1.42	1.36	1.57	1.36

FEV_1: forced expiratory volume in one second; $\dot{V}_{40p}$: expiratory flow at 40% of forced vital capacity.

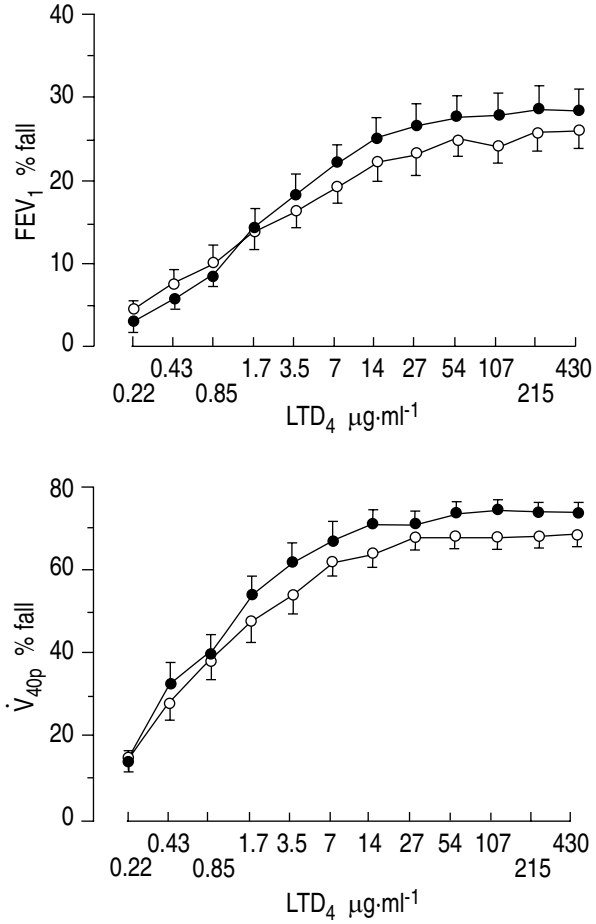

Fig. 1. – Mean (±SEM) concentration-response curves to inhaled LTD_4 for FEV_1 and $\dot{V}_{40p}$ after pretreatment with placebo (open symbols) or thiorphan (closed symbols) in 16 nonasthmatic males. LTD_4: leukotriene D_4; FEV_1: forced expiratory volume in one second; $\dot{V}_{40p}$: expiratory flow at 40% of forced vital capacity.

pretreatment (mean difference ±SD: 5.7±8.1% fall; p=0.013) (fig. 2). Thiorphan also induced a slight increase in $MFEV_1$, which almost reached significance when compared to placebo (mean difference ±SD: 2.7+5.3% fall; p=0.057) (fig. 2). Three of the 16 subjects failed to reach a $PC_{20}FEV_1$ on one occasion, despite administration of the highest concentration of LTD_4 (430 ug·ml^{-1}). In the analysis, these values of $PC_{20}FEV_1$ >430 µg·ml^{-1} were considered as PC_{20}=430 µg·ml^{-1}. There was no significant difference in $PC_{20}FEV_1$ or $PC_{40}\dot{V}_{40p}$ between thiorphan and placebo pretreatments (mean difference±SD (in doubling concentrations): -0.99±1.95 and 0.17±0.95, respectively; p>0.06).

Effect of thiorphan versus *placebo on baseline lung function in asthmatic subjects*

FEV_1 was not different between placebo and thiorphan both before and after pretreatment (table 4) (p>0.58). FEV_1 was slightly, though significantly, decreased by both pretreatments (mean difference ±SD: 0.12±0.16 (*l*) and 0.10±0.14 (*l*), respectively; p<0.04). However, these changes in FEV_1 were not significantly different between

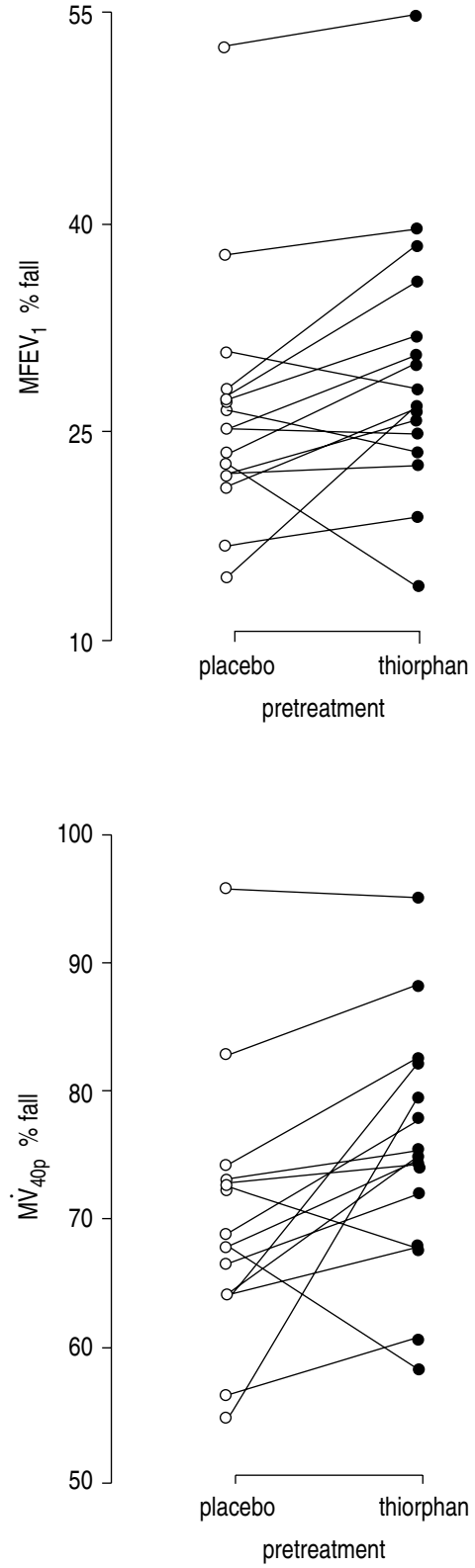

Fig. 2. – Effect of placebo (open symbols) and thiorphan (closed symbols) on $MFEV_1$ and $M\dot{V}_{40p}$ to inhaled LTD_4 in 16 nonasthmatics. $M\dot{V}_{40p}$ increased significantly after thiorphan as compared to placebo pretreatment (p=0.013). The difference in $MFEV_1$ between the two pretreatments failed to reach significance (p=0.057). $MFEV_1$: maximal response to LTD_4 in forced expiratory flow in one second; $M\dot{V}_{40p}$: maximal response to LTD_4 in expiratory flow at 40% of forced vital capacity; LTD_4: leukotrience D_4.

Table 4. – Individual values of baseline FEV_1 and $\dot{V}_{40p}$ before and after placebo or thiorphan in asthmatic subjects

Subject no.	Baseline FEV_1 *l*				Baseline $\dot{V}_{40p}$ $l \cdot s^{-1}$			
	Placebo		Thiophan		Placebo		Thiorphan	
	Before	After	Before	After	Before	After	Before	After
1	3.43	3.51	3.19	3.24	1.35	1.38	1.18	1.38
2	4.02	3.97	4.10	4.23	2.05	2.00	2.65	3.25
3	2.98	2.58	3.12	2.93	1.73	1.20	1.80	1.53
4	4.08	3.89	4.14	3.97	2.93	2.33	2.83	2.83
5	4.09	4.02	4.47	4.19	1.53	1.53	2.40	2.18
6	4.28	4.19	4.23	4.10	2.53	2.25	2.38	2.43
7	3.17	3.25	3.17	3.19	1.58	1.80	1.78	1.63
8	4.40	4.40	3.90	3.69	2.75	2.65	2.05	1.73
9	5.97	5.75	5.89	5.52	6.80	6.00	6.20	6.10
10	4.82	4.77	5.04	5.13	3.03	3.20	3.50	4.00
11	6.47	6.24	6.16	5.87	6.13	5.15	6.15	5.28
12	4.63	4.58	4.47	4.46	3.38	3.43	2.88	2.35
Mean	4.36	4.26	4.32	4.21	2.98	2.74	2.98	2.89
SD	1.04	1.01	0.99	9.92	1.76	1.50	1.61	1.52

For abbreviations see legend to table 3.

placebo and thiorphan (p>0.70). Post-pretreatment FEV_1 was not different between the two pretreatments (p>0.57). $\dot{V}_{40p}$ was not affected by either thiorphan or placebo pretreatment (table 4) (p>0.06) and the difference in the changes in $\dot{V}_{40p}$ between the two pretreatments were non-significant (p>0.25). Post-pretreatment $\dot{V}_{40p}$ was also not different between the two pretreatments (p>0.46) (table 4).

Effect of thiorphan versus *placebo on the concentration-response curve to LTD_4 in asthmatic subjects*

Only 4 out of 12 asthmatic subjects reached a maximal-response plateau to LTD_4. Thiorphan failed to shift the concentration-response curve to LTD_4. There was no significant difference in $PC_{20}FEV_1$ or $PC_{40}\dot{V}_{40p}$ between thiorphan and placebo pretreatment (mean difference ±SD (in doubling concentrations): -0.19±1.23 and 0.12±0.73, respectively; p>0.57).

Discussion

The results of the present study indicate that NEP inhibition by thiorphan enhances maximal airway narrowing to inhaled LTD_4 in humans *in vivo*. This potentiating effect was rather small, and could only be detected in the normal subjects using a sensitive index of airway narrowing ($\dot{V}_{40p}$). We were unable to evaluate this finding in the asthmatic subjects, since, not unexpectedly, most of them failed to reach a maximal-response plateau to inhaled LTD_4. The sensitivity to LTD_4 was not changed by thiorphan in either group of subjects. Based on the evidence from animal studies that NEP inhibition induces unopposed activity of tachykinins [14, 21, 22], thesc findings favour the hypothesis that cysteinyl leukotrienes may exert their bronchoconstricting effects, in part, by secondary release of endogcnous neuropeptides in human airways *in vivo*.

This is the first study on the interaction of LTD_4 and endogenous neuropeptides in human airways *in vivo*. The findings of our study confirm and extend the results of previous *in vitro* observations. Release of endogenous neuropeptides (substance P, neurokinin A) has been demonstrated in guinea-pig ileum and trachea following administration of LTD_4 [13, 16]. In addition, the NEP inhibitors, thiorphan and phosphoramidon, have been reported to potentiate LTD_4-mediated release of substance P in isolated perfused guinea-pig lungs [14], and to enhance LTC_4-induced contractile response in bronchial strips of guinea-pigs [15], respectively. Therefore, the present finding of enhanced LTD_4-induced bronchoconstriction by pretreatment with thiorphan in humans *in vivo* is consistent with the results in animal studies.

In the present study, we examined the effects of inhaled thiorphan on the concentration-response curve to inhaled LTD_4 in both normal and asthmatic subjects. We do not believe that our findings can be explained by measurement errors, since our data have been obtained by means of validated methodology [6, 22]. Using similar thiorphan pretreatment to a provocation test with neurokinin A, CHEUNG and co-workers [22, 23] have recently provided indirect evidence of endogenous NEP activity in normal and asthmatic humans *in vivo*. Taking into consideration the possibility of differences in LTD_4 effects or NEP activity based on the presence of atopy and/or asthma, we purposely selected two groups of subjects consisting of nonsmoking, nonatopic normals, and of nonsmoking, atopic subjects with clinically stable, mild asthma. Based on current literature, the sample size of both groups of subjects was large enough to detect sufficiently small differences in sensitivity and maximal response to LTD_4 [28, 29]. In the present study, we found that the reproducibility of the LTD_4 challenges was good: there was no difference in $\ln PC_{20}FEV_1$ or $\ln PC_{40}\dot{V}_{40p}$ between screening and placebo day (mean difference ±SD: 0.17±0.90 and 0.08±0.69, respectively; p>0.7). Since LTD_4 is a very expensive compound, we did not go as far as the maximal-response plateau in the normal subjects on the screening day. When recalculating the sample size from

the present data, the power of the analysis was sufficient, detecting a change in $PC_{40}\dot{V}_{40p}$ of 0.88 and 1.04 in the normal and asthmatic group, respectively. The subjects visited the laboratory at the same time ±2 h of the day, and a wash-out interval of two weeks was considered to be sufficient, based on the fact that FEV_1 was within 10% on both days preceding the LTD_4 challenges.

Leukotrienes do not seem to be unique in their capacity to induce release of endogenous neuropeptides. Several experimental studies have demonstrated that various exogenous irritants, including inflammatory mediators, such as bradykinin [30, 31], methacholine [16], histamine [16, 31], and platelet-activating factor [16], may cause release of neuropeptides in airway tissue. This may include the release of the neuropeptide calcitonin gene-related peptide (CGRP) and tachykinins, such as substance P (SP), neurokinin A (NKA), and neurokinin B (NKB), which are considered to be responsible for excitatory NANC responses [18–20]. According to recent evidence, chemical or mechanical stimulation of sensory C-fibres may produce local axon reflexes which may result in the release of endogenous neuropeptides [18, 31, 32]. In the airways, these neuropeptides are able to increase the tone of bronchial smooth muscle, to enhance microvascular permeability, mucus secretion, epithelial chloride transport, and cholinergic neurotransmission, and to activate inflammatory cells, producing so called "neurogenic inflammation" [18–20]. Normally, neuropeptides are degraded by at least two enzymes: angiotensin converting enzyme (ACE, EC 3.4.15.1, kinase II), which cleaves intravascular peptides, and neutral endopeptidase (NEP, EC 3.4.24.11, enkephalinase), which appears to be the most important enzyme for the modulation of neurogenic inflammatory responses both at sites of neuropeptide release and at sites of neuropeptide action [18, 21, 33]. NEP is involved in the degradation of substances with bronchoconstrictor properties, such as the neuropeptides substance P and neurokinin A [21], and the oligopeptide endothelin produced by endothelial cells, which appeared to be a potent vasoconstrictor and constrictor of airway smooth muscle [34]. Moreover, NEP also degrades peptides with bronchodilator properties, such as vasoactive intestinal peptide (VIP) [35], and atrial natriuretic factor (ANF) [36]. Thiorphan effectively blocks NEP and ACE and, therefore, potentiates the effects of endogenous neuropeptides [21, 37].

How can we explain the enhanced maximal airway narrowing to LTD_4 after pretreatment with thiorphan? There is accumulating evidence that maximal airway narrowing is determined mainly by inflammatory changes in the bronchial wall, predominantly caused by plasma exudation and oedema [38–40]. This is in keeping with the observation that the bradykinin-induced increase in maximal response to acetylcholine in feline airways *in situ* is associated with the degree of microvascular leakage [41]. Regarding sensory neuropeptides, these effects are considered to be particularly induced by NK_1 receptors [20] and CCRP receptors [18–20]. Although these findings have not yet been confirmed in human studies, the observations of the present study suggest that the potentiating effect of thiorphan on maximal airway narrowing to inhaled LTD_4 may be caused predominantly by the release of SP and CCRP. Moreover, NKA and NKB also stimulate NK, receptors, although in a less potent manner [20]. It could be speculated that these mechanisms predominate in asthma, since OLLERENSHAW *et al.* [42] reported an increase in both number and length of SP-immunoreactive nerve fibres in airways of asthmatic subjects, as compared to those of nonasthmatic subjects. However, we could not provide functional evidence for this, because of the lack of maximal-response plateaus to LTD_4 in the majority of the asthmatic subjects. It is clear, that a definite conclusion about the mechanism of the potentiating effect of thiorphan on LTD_4 can only be obtained by direct measurement of neuropeptides in the bronchoalveolar lavage (BAL) or by administration of potent and specific neurokinin receptor antagonists.

The observation that an inflammatory mediator of asthma, such as LTD_4, produces secondary release of sensory neuropeptides may have clinical implications. It could be argued that therapeutic strategies of asthma could also be directed towards prevention of the release of neuropeptides and the suppression of neurogenic inflammation [21]. This may already be achieved by inhaled steroids [43], which have been observed to reduce the maximal degree of airway narrowing in asthma [40]. In the future, neurogenic inflammation may also be limited by neurokinin receptor antagonists or by recombinant NEP suppletion [21, 30]. However, before these strategies can be applied, the role of endogenous release of neuropeptides in response to naturally occuring stimuli, such as allergens or exercise, needs to be elucidated.

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