

Experimental Rhinovirus Infection Increases Human Tissue Kallikrein Activation in Allergic Subjects

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Key Words

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Abstract

Background: Rhinovirus infection is a major cause of asthma exacerbations. While rhinovirus infection is known to generate kinins in the upper respiratory track, little is known about the effect of rhinovirus on kinin generation in the lower airway. We previously identified human tissue kallikrein (hTK) as the principal lung kininogenase during allergic airway inflammation. In this report we investigate the effect of experimental rhinovirus infection on hTK activity in the airways of atopic subjects with and without asthma. **Methods:** Eight atopic subjects, 4 with asthma, underwent bronchoscopy with lavage. At least 1 month later, subjects were inoculated with rhinovirus, then underwent repeat bronchoscopy with lavage 4 and 18 days later. hTK mRNA was measured in nasal scrape samples by quantitative real-time PCR. hTK activity (chromogenic substrate assay) and IL-8 levels (ELISA) were assessed in the bronchoalveolar lavage fluid. **Results:** At day 4 after rhinovirus inoculation, nasal hTK mRNA was modestly increased in both the rhinitis (1.7-fold) and asthmatic (2.1-

fold) groups. A doubling or greater increase in hTK activity after rhinovirus infection was observed in all 4 asthmatic subjects (mean 19-fold increase) but only in 1 of 4 atopic subjects without asthma (mean 2-fold increase). Rhinovirus infection also increased the IL-8 protein level in bronchoalveolar lavage fluid, which correlated with hTK activity ($R = 0.82$). **Conclusion:** Experimental rhinovirus infection in allergic asthmatic subjects is accompanied by increased lower airway hTK activation, which parallels the appearance of IL-8. Rhinovirus-induced hTK activation may contribute to airway inflammation and asthmatic exacerbations.

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Introduction

Rhinovirus is a recognized culprit for the most frequent acute respiratory illness in humans, the common cold. In addition to its upper respiratory effects, rhinovirus infections have unique consequences for individuals with asthmatic disease and are a major trigger for epi-

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sodic asthma exacerbations, which dramatically enhance disease-related morbidity [1]. Utilizing a variety of in vitro and in vivo models as well as natural infections, investigators have made substantial progress in defining multiple discrete inflammatory effects of rhinovirus infection that could impact the airway [for review, see 2]. Rhinovirus has been shown to be capable of propagating in the lower airway [3], and the ability of rhinovirus to induce inflammatory cytokine expression was recently shown to depend on NF- κ B activation and viral replication [4]. As airway inflammation is generally accepted to be the primary determinant of clinical disease severity in asthma, the interrelationship between viral infections and inflammatory cascades is of significant interest.

In human subjects experimentally infected with rhinovirus, upper respiratory symptom severity directly correlates with the level of kinin generation measured in nasal secretions [5]. There is no human in vivo information, however, pertaining to the impact of rhinovirus infection on the kallikrein-kinin system in the lower airway. We previously reported that kallikrein, the enzyme responsible for the generation of kinins, is activated and kinin generated during natural and experimental allergic inflammation in the lower airways of asthmatic subjects [6, 7]. Furthermore, these studies established that the kininogenase activity in bronchoalveolar fluid reflected tissue kallikrein (TK) activity and not plasma kallikrein activity [6]. Here we hypothesize that rhinovirus infection induces an increase in human TK (hTK) activity in the lower airway, which is more pronounced in asthmatic subjects compared to nonasthmatic subjects. To address this we measured and compared the level of hTK activity, as well as the level of the pro-inflammatory cytokine IL-8 as a marker of inflammation, in the bronchoalveolar lavage (BAL) fluid from atopic asthmatics and atopic nonasthmatic subjects before and after experimental rhinovirus infection.

Material and Methods

Volunteer Subjects and Clinical Protocol

Nonsmoking adults with allergic rhinitis with or without asthma were recruited for the study. All gave informed consent in accordance with the protocol approved by the institutional review board. Eight atopic subjects, 4 of whom also had asthma, participated in the study. Asthmatic subjects were restricted to intermittent or mild persistent asthma ($FEV_1 > 80\%$ of predicted). All subjects underwent allergy skin testing and methacholine inhalation challenge to assure accurate classification of atopic and asthmatic status. Blood was drawn at the initial visit to measure rhinovirus 16-neutralizing antibodies. All subjects with antibody titers > 1.2 were excluded.

An initial baseline bronchoscopy with lavage was taken at least 4 weeks prior to rhinovirus inoculation. Nasal samples were collected by gentle scraping of the inferior turbinate using a Rhino-probe curette (Arlington Scientific Inc., Springville, Utah, USA) as previously described [8]. Nasal rhinovirus 16 challenge was performed as described previously by Calhoun et al. [9]. Briefly, subjects were inoculated with a rhinovirus 16 suspension (0.25 ml) containing 500–18,000 tissue culture-infective dose 50% in each nostril on days 1 and 2. After inoculation and at days 4 and 18, a nasal scrape followed by a bronchoscopy with lavage was performed. The postinoculation samples at days 4 and 18 were taken from separate (either right or left) lungs to minimize the influence of the bronchoscopy procedure.

Measurement of hTK Activity in BAL

A chromogenic substrate for glandular kallikreins, H-D-Val-Leu-Arg-pNA, was utilized to kinetically examine the amidolytic activity of the BAL kininogenase. H-D-Val-Leu-Arg-pNA (S-2266; KabiVitrum, Stockholm, Sweden) was diluted in buffer (0.05 M Tris, pH 8.0) to achieve a concentration of tripeptides of 200 μ M. Sixty microliters of BAL was added to 150 μ l of tripeptide solution in a 96-well microtiter plate. Controls included tripeptide alone and 12 ng of purified human urinary kallikrein (KLK1). The reaction was incubated at room temperature and activity was determined by measurement of the change in optical density (OD) per time unit at 405 nm.

Measurement of IL-8 Protein in BAL

IL-8 was measured using paired antibody quantitative enzyme-linked immunosorbent assays (ELISA) and appropriate blanks following the manufacturer's directions (R&D Systems, Abingdon, UK).

Measurement of hTK mRNA in Nasal Scrapings

Nasal scrapings were taken at each time point and assayed for hTK mRNA and housekeeping gene β -actin by quantitative real-time PCR as described previously [8]. Briefly, total RNA from nasal scrape samples was isolated, reverse transcribed and assessed by quantitative real-time PCR. The primers used were as follows: hTK forward primer: 5'-ctgtcgcgcagagtgctg-3'; hTK reverse primer: 5'-ggctggcgcttcaggagttc-3'; β -actin forward primer: 5'-tgctgacattaaggagaag-3'; β -actin reverse primer: 5'-gtcagcgagctcgtagctct-3'. The following dual-labeled probes were obtained from BioSearch Technologies (Novato, Calif., USA): β -actin 5' FAM-caggctgcttcaggctctc-3' BHQ1 and hTK 5' FAM-tccgctatgtgtcctcgtcacttcaca-3' BHQ1.

Statistical Analysis

Data were analyzed by the nonparametric two-tailed Mann-Whitney U test. $p < 0.05$ were considered significant.

Results

Clinical Response to Experimental Rhinoviral Infection

Subjects were given questionnaires to record postinoculation symptoms and severity. All subjects developed

Table 1. Clinical characteristics of subjects

Dx	Sex	Age	Asthma severity	ST	RV inoculation	RV PCR	Postviral inoculation symptoms		Medications	1st FEV ₁	2nd FEV ₁	3rd FEV ₁	Max TK
							severity	description					
R	M	34	NA	grass, cat	yes	+	moderate	sore throat, nasal congestion, nasal drainage, snoring, cough	none	103	90	92	0.35
R	M	20	NA	mite, grass	yes	+	moderate	nasal congestion, nasal drainage, snoring, aching, fever, malaise, cough, chest congestion, wheeze	none	85	85	85	6.00
R	M	32	NA	mite, tree	yes	+	mild	malaise, wheeze	bupropion, venlafaxine	116	116	109	1.45
R	M	26	NA	mite, cockroach	yes	+	moderate	sore throat, nasal congestion, nasal drainage, snoring, aching, fever, malaise, headache, cough	paroxetine	100	101	97	1.71
R+A	M	25	M-P	grass, dog, cat, mite	yes	+	moderately severe	sore throat, nasal congestion, cough, nasal, increased sputum	albuterol, inhaled fluticasone	96	103	100	4.81
R+A	F	25	M-I	grass, cockroach	yes	+	mild	headache, sore throat, aching	albuterol	97	102	103	57.50
R+A	F	25	M-P	grass	yes	+	mild	itchy nose, watery eyes, aching	albuterol, inhaled triamcino-lone, salmeterol, BCP	100	102	95	9.25
R+A	F	23	M-I	cat, grass	yes	+	moderate	sore throat, nasal congestion, nasal drainage, sneeze, chest congestion	albuterol, inhaled beclomethasone, loratadine, guaifenesin/phenylephrine, BCP	89	89	90	5.75

Skin testing (ST) reports allergens that gave a positive skin test. Symptom severity reflects subjects' overall assessment of his/her symptoms. FEV₁ values were measured prior to each of the bronchoscopy procedures as identified. Dx = Diagnosis; RV = rhinovirus; Max TK = maximal fold increase in TK activity after rhinovirus infection compared to baseline; R = allergic rhinitis; NA = not applicable; A = asthma; M-P = mild persistent; M-I = mild intermittent; BCP = birth control pills.

mild to moderate symptoms that were transient (lasting less than 3 days) and typically consisted of nasal congestion, throat irritation, malaise and increased mucoid secretions (table 1). Experimental rhinoviral infection was confirmed by RT-PCR detection of rhinovirus RNA in nasal scrapes of all subjects 4 days following inoculation. None of the subjects reported a significant exacerbation of asthma symptoms following rhinovirus inoculation, and the FEV₁ was unchanged from baseline at the time of repeat bronchoscopy at days 4 and 18.

Experimental Rhinovirus Infection Induced an Increase in hTK Activity in the Atopic Human Airway

To assess whether hTK activity changed during experimental rhinovirus infection, we measured hTK activity in BAL before and after nasal rhinovirus inoculation. BAL fluid was assessed for hTK activity by examining the amidolytic activity of BAL kininogenase using a chromogenic substrate for glandular kallikreins. BAL activity was measured as the rate of change in OD measured at 405 nm. At baseline, there was no difference in the hTK activity between the atopic asthmatic (0.006 ± 0.003)

and atopic nonasthmatic (0.007 ± 0.003) subjects; however, following rhinovirus challenge, the atopic asthmatic subjects showed enhanced hTK activity (day 4, 0.039 ± 0.014 ; day 18, 0.030 ± 0.019) compared to the atopic nonasthmatic subjects (day 4, 0.016 ± 0.022 ; day 18, 0.007 ± 0.003).

The data were also expressed as the maximum fold change in kininogenase activity from before to after rhinovirus inoculation, allowing each subject to be his/her own control. Experimental rhinovirus infection increased BAL fluid hTK activity. A doubling or greater increase in hTK activity after rhinovirus infection was observed in all 4 asthmatic subjects (mean 19-fold increase), but only in 1 of 4 nonasthmatic atopic subjects without asthma (mean 2-fold increase; fig. 1). Comparing the hTK levels at both time points, there was a statistically significant increase in hTK activity in the asthmatic group ($p = 0.01$).

We also measured hTK mRNA in nasal scrape samples prior to and 4 days following experimental rhinovirus inoculation. A consistent but modest increase in hTK mRNA expression was seen in all subjects except for 1 of

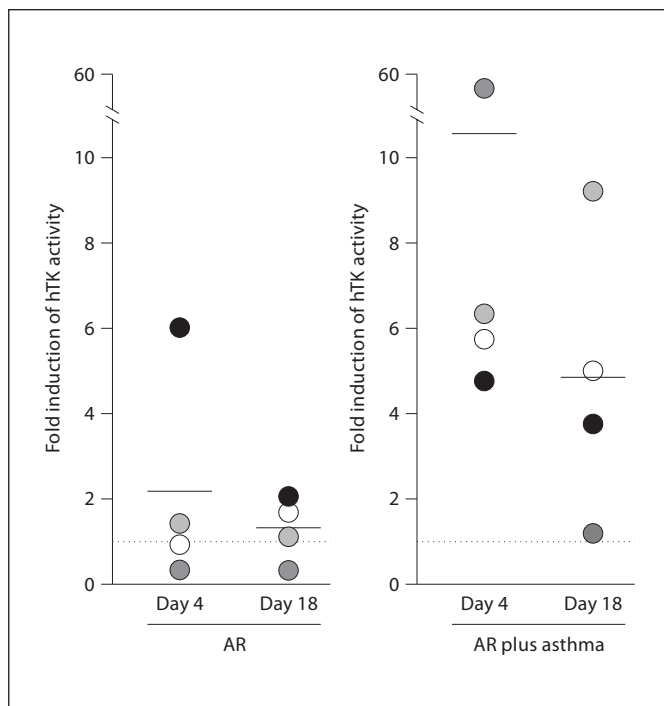


Fig. 1. hTK activation is increased following experimental rhinovirus infection. The fold induction of hTK activity measured by change in OD at 405 nm following amidolytic cleavage of H-D-Val-Leu-Arg-pNA chromogenic substrate is displayed for atopic subjects with allergic rhinitis with and without asthma at days 4 and 18 compared to the preinfection baseline values. Shading identifies individual patients. AR = Allergic rhinitis.

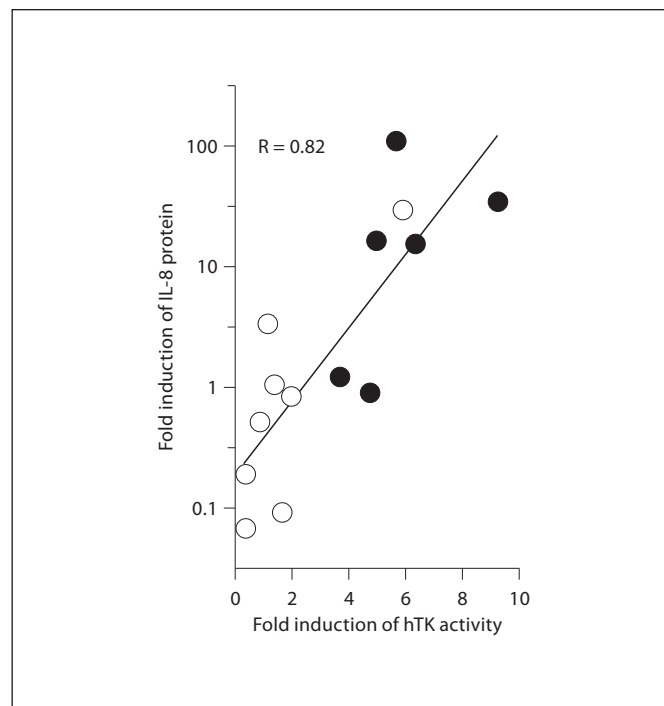


Fig. 2. IL-8 protein increases following experimental rhinovirus infection. Fold induction of IL-8 protein measured by ELISA (logarithmically transformed) versus fold induction of hTK activity is displayed for 14 paired samples taken from subjects at days 4 and 18 compared to the preinfection baseline values. ○ = Allergic rhinitis; ● = allergic rhinitis with asthma.

the atopic subjects. The average induction of nasal hTK mRNA in the asthmatic group (2.1-fold) was not significantly different than the induction in the atopic group (1.7-fold, $p = 0.95$).

Rhinovirus-Induced hTK Activity Correlates with Rhinovirus-Induced IL-8 Protein

We next assessed the effect of experimental rhinovirus infection on levels of IL-8 protein in BAL fluid and its relationship to the changes in hTK activity. The amount of IL-8 protein was measured by ELISA in BAL fluid before and after rhinovirus inoculation in 7 subjects (3 with asthma).

IL-8 levels were increased by a mean induction of 8.5-fold in the BAL fluid of atopic subjects without asthma following rhinovirus infection. In the atopic subjects with asthma, IL-8 protein levels were increased to a significantly greater degree in the BAL fluid following rhinovirus infection by a mean induction of 50-fold ($p =$

0.03). Interestingly, after rhinovirus infection the level of IL-8 protein correlated with the level of hTK activity ($R = 0.82$; fig. 2).

Discussion

In this report we demonstrate that experimental rhinovirus infection in atopic subjects leads to increased BAL hTK activity in vivo. The increase in hTK activity was significantly more robust for atopic subjects with asthma than for atopic subjects without asthma. In comparison, there was no difference in the extent to which rhinovirus infection induced nasal hTK mRNA in the asthmatic and atopic subjects. These findings are of particular interest given the exaggerated lower airway morbidity observed in asthmatic cohorts following rhinovirus infections [1]. While the mechanism whereby rhinovirus infection increased hTK activity was not explored, activa-

tion of hTK in the airway has been linked to degradation of the glycosaminoglycan hyaluronan, which immobilizes hTK and inhibits its catalytic activity [10]. As hyaluronan is depolymerized by reactive oxygen species, increased hTK activity would be expected in diseases associated with increased airway reactive oxygen species production – such as rhinovirus infection in asthmatics.

Activation of hTK in human airways results in enhanced kinin generation [6, 7]. In both the upper and lower airway, bradykinin will provoke symptoms which mimic naturally occurring disease, including nasal congestion, drainage and throat irritation following nasal inhalation and in asthmatic but not normal subjects' cough, chest tightness, as well as measurable drops in flow rates. Subjects with allergic rhinitis but not nonallergic rhinitis have been shown to be hypersensitive to nasal bradykinin challenge [11]. The importance of kinin generation in asthma is emphasized by the beneficial effects of kinin antagonists [12, 13]. Treatment with an inhaled B₂R antagonist (HOE-140) results in a gradual dose-dependent increase in FEV₁ for moderate asthmatics, suggesting an anti-inflammatory effect resulting from the B₂R blockade [14]. The effects of kinin generation in asthmatic subjects during viral infection may be further exaggerated, as experimental rhinovirus 16 infection abolished the typical tachyphylaxis to bradykinin challenge in asthmatic subjects [15]. hTK may also exert an important influence on the pathogenesis of airway inflammation independent of kinin generation. TK, generated by oxidative stress in a primary human trachea culture model,

was able to process proepidermal growth factor into bioactive epidermal growth factor, a factor that can induce submucosal gland hypertrophy, hyperplasia and mucous hypersecretion [10].

In the current study, protein levels of IL-8 in BAL fluid correlated with the degree of hTK activation following rhinovirus infection. IL-8 has been previously linked with kinin generation following experimental allergen challenge in the upper airway. In vivo, the bradykinin antagonist HOE-140 abrogated allergen-induced increases in nasal lavage fluid IL-8 along with histamine hyperresponsiveness as well as the appearance of eosinophils, ECP and kinin, in allergic rhinitis subjects [16].

In conclusion, this study provides the novel observations that lower airway hTK activity is increased in vivo following experimental rhinovirus infection in atopic subjects. In those atopic subjects with a history of asthma, there was a disproportionate increase in hTK activity. A positive correlation was observed between the levels of the inflammatory chemokine IL-8 and hTK activity. These findings, along with previous work, support a potential role for the kallikrein-kinin system in rhinovirus-induced asthma exacerbations.

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