Thorax Online First, published on June 3, 2009 as 10.1136/thx.2009.113183

Nerve growth factor enhances cough and airway obstruction via TrkA and TRPV1 receptor - dependent mechanisms

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Funding: Kuwait University Research Administration, Grant Number PT01/05
Running title: NGF enhances cough via TrkA and TRPV1 receptors
Keywords: Nerve growth factor, cough, airway obstruction, TrkA, TRPV1
Word count for body of manuscript: 3937

ABSTRACT

Background: Nerve growth factor (NGF) is an important mediator of airway hyperresponsiveness and hyperalgesia but its role in cough is unknown.

Objectives: In this study we investigated the effects of NGF on the cough reflex and airway caliber in guinea pigs. We also assessed the involvement of the tropomyosin-related kinase A (TrkA) and transient receptor potential vanilloid-1 (TRPV1) receptors, and p38 mitogen activated protein kinase (MAPK) - dependent pathway on any NGF-induced effects on cough and airway obstruction.

Methods: Guinea pigs were placed in a transparent whole body plethysmograph box. Cough was assessed visually, acoustically and by analysis of the flow signal. Airway obstruction was measured using enhanced pause (Penh) as an index.

Results: Exposure of guinea pigs to NGF did not induce a cough response nor a significant airway obstruction. However, exposure of guinea pigs to NGF immediately before citric acid inhalation resulted in a significant increase in the citric acid-induced cough and airway obstruction compared to vehicle treated animals. Pre-treatment with the TrkA receptor antagonist, K252a, or the TRPV1 antagonist, iodoresiniferatoxin, significantly inhibited the NGF enhanced cough and airway obstruction. Exposure to NGF also increased p38 MAPK phosphorylation, but pretreatment with the p38 MAPK inhibitor, SB203580, did not affect either the NGF enhanced cough or airway obstruction despite preventing the NGF-induced elevation in p38 MAPK phosphorylation.

Conclusions: The data show that NGF can enhance both cough and airway obstruction via a mechanism that involves the activation of TrkA and TRPV1 receptors but not the p38 MAPK - dependent pathway.

INTRODUCTION

Cough has a high prevalence rate and is one of the most common symptoms for which sufferers seek medical help from primary care physicians and pulmonologists [1].

Several pharmacological receptors have been identified to be important in mediating cough [2, 3]. Currently however, most of the mechanistic information on cough is essentially derived from animal models with a normal cough reflex where a baseline type cough is induced through the use of chemicals such as citric acid or capsaicin [4, 5]. Although this approach is very useful in dissecting out the airway cough receptors and those gating the cough center, we also need to address major issues with some types of clinical cough such as the enhanced cough reflex which is seen in acute cough associated with upper respiratory tract infections (URTI) [6]. Hence, mediators involved in lowering the cough threshold and the mechanisms involved need to be studied. Models of chronic spontaneous cough associated with URTI, gastro-esophageal reflux and asthma are also needed to improve the understanding of the mechanisms underlying this type of cough.

Nerve growth factor (NGF) is a member of the neurotrophin family of proteins that are known to regulate neuronal development and maintenance, and are also involved in the sensitization of spinal circuitry that underlies many forms of hyperalgesia [7]. The biological activity of NGF is thought to be mediated by two main receptors: the high affinity tropomyosin-related kinase A (TrkA) containing an intrinsic tyrosine kinase activity and a low affinity receptor p75 for neurotrophins (NTR) [8].

The notion that NGF may be an important mediator of enhanced cough is implied by studies highlighting its importance in airway hyperresponsiveness and hyperalgesia; two phenomena that share many similarities with cough [9, 10]. For example, NGF has been shown to induce airway hyperresponsiveness *in vivo* in guinea pigs [11] and mice [12, 13] and *in vitro* in human bronchus [14]. Also, NGF was reported to be an important mediator in both primary and secondary hyperalgesia [15, 16]. Although both NGF receptors appear to have a role in hyperalgesia, there is stronger evidence for TrkA receptor involvement [17]. There is also evidence to suggest that NGF sensitization of nociceptive neurons is mediated via the activation of TRPV1 [18] and that both p38 MAPK and PI3K dependent pathways seem to couple the activation of TrkA receptors to TRPV1 stimulation [18, 19]. A recent clinical study did not find a significant increase in NGF levels in either serum or sputum in patients with chronic cough. However, it did report a significant correlation between the duration of cough and NGF sputum levels suggesting a complex relationship between cough and NGF [20]. NGF levels have also been reported to be elevated in asthma [21] and in allergic rhinitis following allergen provocation [22] suggesting a possible role for this mediator in some aspects of respiratory disease.

In order to study the effect of NGF on the cough reflex and airway caliber we investigated 1), whether NGF can enhance the citric acid-induced cough and airway obstruction 2), the role of TrkA and TRPV1 receptors as well as the p38 MAPK - dependent pathway in any NGF mediated effects on the cough reflex and airway obstruction.

METHODS

Animals

Dunkin-Hartley guinea pigs (300-600 g) of either sex were used in this study. The care and use of animals was approved by the Animal Welfare and Use of Laboratory Animals Committee in the Health Sciences Center, Kuwait University. All animals were randomly assigned into control and experimental groups.

Measurement of cough response

The animals were continuously watched by a trained observer who counted the number of coughs. Coughs were recognized by the characteristic animal posture and a rapid transient increase in airflow over and above the normal flow. Also, the criteria for cough included a high sound with the mouth open and a defined pattern in the sound signal which distinguishes coughs from sneezes.

Measurement of airway obstruction

Airway function was measured using whole body plethysmography. For more details refer to the online supplement.

Protein isolation from the lungs and western blot analysis

For details refer to the online supplement.

Compounds

Chemicals used were citric acid, phosphate buffered saline (PBS) tablets, bovine serum albumin (BSA), SB203580, nerve growth factor (NGF-7S mouse submaxillary glands) and α-actin monoclonal antibody (Sigma-Aldrich, Germany), K252a (Fluka BioChemika, Switzerland), 5´ Iodoresiniferatoxin (IRTX) (Tocris, Cookson Ltd., Langford, U.K), dimethyl sulfoxide (DMSO) (BDH Laboratory), nitrocellulose membranes (Biorad laboratories, USA), ECL kit, primary and secondary antibodies (Amersham, UK). Stock solutions of NGF (50 µg/ml) in 1% BSA in PBS were stored in aliquots at -80 ºC and used as freshly prepared dilutions of 0.3 and 1 µg/ml. Stock solution of K252a was made by dissolution in 30% DMSO (in normal saline); subsequent dilutions were made in normal saline. Stock solutions of IRTX and SB203580 were made by dissolution in 100% DMSO; subsequent dilutions were made in distilled water. All the stock solutions were kept in aliquots at -80 ºC and dilutions were made on the day of the experiment. Citric acid (0.2 M) was prepared freshly in PBS.

Experimental protocol

Effect of immediate and 24 hr post NGF aerosolization on citric acid-induced cough and airway obstruction in guinea pigs

Five groups were randomly assigned. Groups 1 (n=10) and 2 (n=14) received NGF (0.3 and 1 µg/ml, respectively) by inhalation over 10 min and were then immediately given 0.2 M inhaled citric acid. Two control groups (3 and 4) were set-up. Group 3 (n=14) received BSA (in PBS) which was the vehicle for NGF. Group 4 (n=10) received PBS which was the vehicle for BSA (the latter group was established so as to ascertain whether BSA had any effect on cough or airway obstruction). Immediately following exposure to either BSA or PBS, animals were given 0.2 M inhaled citric acid. Group 5 (n=10) received NGF (1 µg /ml) and 24 hr later the animals were given 0.2 M inhaled citric acid. Cough and airway obstruction were assessed in all groups during the citric acid challenge and 5 min thereafter.

Effect of the TrkA receptor antagonist, K252a (20 µg/kg), on NGF enhanced citric acid-induced cough and airway obstruction

Two groups were randomly assigned. Group 1 (n=8) was pretreated with the vehicle and group 2 (n=9) was pretreated with K252a (20 µg/kg, i.p.). A dose of 20 µg/kg of the inhibitor K252a was chosen as a similar dose was previously reported to significantly inhibit the development of anaphylactic shock in guinea pigs [23]. After 30 min, animals from both groups received inhaled NGF (1 µg/ml) for 10 min. Immediately following NGF exposure, animals were exposed to inhaled citric acid (0.2 M) for 10 min. Both cough and airway obstruction were assessed during the citric acid challenge and 5 min thereafter.

Effect of the TRPV1 antagonist, IRTX (7.5 µg/ml), on NGF enhanced citric acid-induced cough and airway obstruction

Two groups were randomly assigned. Group 1 (n=8) was pretreated with the vehicle and group 2 (n=9) was pretreated with IRTX (7.5 µg/ml). A dose of 7.5 µg/ml IRTX was chosen as a similar dose was previously reported to have inhibitory effects on cough [4]. Both vehicle and drug were administered by aerosol over 10 min. Immediately following exposure to IRTX or its vehicle, the animals received inhaled NGF (1 µg/ml) for 10 min. At the end of the NGF exposure, the animals were exposed to inhaled citric acid (0.2 M) for 10 min. Both cough and airway obstruction were assessed during the citric acid challenge and 5 min thereafter.

Effect of the p38 MAPK inhibitor, SB203580 (22.6 µg/ml), on NGF enhanced citric acid-induced cough and airway obstruction

Two groups were randomly assigned. Group 1 (n=8) was pretreated with the vehicle and group 2 (n=9) was pretreated with SB203580 (22.6 µg/ml). This dose was based on a study where SB203580 was shown to inhibit NGF-induced phosphorylation of p38 MAPK [18]. Both vehicle and drug were given by aerosol over 10 min. Immediately following exposure to SB203580 or its vehicle, the animals received inhaled NGF (1 µg/ml) for 10 min. At the end of the NGF exposure, the animals were exposed to inhaled citric acid (0.2 M) for 10 min. Both cough and airway obstruction were assessed during the citric acid challenge and 5 min thereafter.

For the protocols using 2 treatment groups, the experiment was typically run twice or three times with an n=2-3 guinea pigs in each treatment group in each run. For the protocol using 5 treatment groups, the experiment was typically run three to five times with an n=2-3 guinea pigs in each treatment group in each run. This ensured that all groups were subjected to the same conditions at the same time and hence reduced variability. Also, all groups had almost equal number of male and female guinea pigs.

Expression of results and statistical analysis:

For the cough experiments, the data are expressed as mean±SEM and represent the number of coughs during a 15 min period. Airway obstruction is represented as an absolute change in Penh and is expressed as mean±SEM. The differences in the degree of the airway obstruction between different groups were determined by calculating the mean Penh value for the last 10 min for each guinea pig. All differences between groups were expressed as mean difference. All the data were first analyzed for normal distribution then tested for statistical difference using, in case of the multiple groups, a one-way analysis of variance (ANOVA). The difference between two treatment groups was analyzed using either

parametric (non-paired t-test) or non-parametric test (Mann-Whitney Rank Sum Test). All statistical analyses were carried out using a statistical programme (SPSS –version 17, USA) and for all studies, probability values of $P < 0.05$ were considered statistically significant.

RESULTS

Effect of immediate and 24 hr post NGF treatment on citric acid-induced cough and airway obstruction in guinea pigs

During the NGF exposure (either 0.3 or 1 μg/ml), no cough response or statistically significant airway obstruction were noted (data not shown). However, approximately 40% of the guinea pigs exposed to NGF (1 μg/ml) did demonstrate a modest degree of airway obstruction compared to BSA or PBS exposed animals (data not shown). Our data show that the citric acid-induced cough in the PBS pretreated animals was low with a mean number of coughs of 1.9±1.2 coughs over 10 min. Pretreatment with NGF aerosol, at 1 μg/ml but not 0.3 μg/ml, induced a statistically significant increase in the mean number of coughs compared to PBS exposed animals with a mean difference of 10.70 ($P<0.001$). This increase in the mean number of coughs was specifically due to the NGF and not the BSA as administration of BSA alone resulted in a cough response similar to that seen in the PBS treated group with a mean difference of 0.23 ($P=0.92$). Indeed, NGF enhanced cough was statistically significantly greater than both the PBS and BSA treated groups (Figure 1A). Furthermore, no enhanced cough was observed 24 hr following exposure to 1 μg/ml NGF compared to the PBS group, the mean difference was 0.40 ($P=0.87$). On the basis of these studies, 1 μg/ml of NGF was selected for subsequent experiments.

Our data also show that the citric acid-induced airway obstruction in the PBS pretreated animals was low with a mean Penh value of 0.9±0.1. Pretreatment with NGF aerosol, at 1 μg/ml but not 0.3 μg/ml, induced a statistically significant increase in the citric acid-induced airway obstruction compared to PBS treated guinea pigs with a mean difference of 4.28 ($P<0.001$, Figure 1B). This increase in the airway obstruction again was specific to the NGF and not the BSA as administration of the BSA alone did not result in a significant airway obstruction compared to that seen in the PBS treated group, the mean difference was 0.36 ($P=0.65$, Figure 1B). Indeed, the NGF (1μg/ml) enhanced airway obstruction was statistically significantly greater than both the PBS and BSA treated groups with a mean difference of 4.28 ($P<0.001$) and 3.92 ($P<0.001$), respectively. Additionally, the NGF (1 μg/ml) enhanced airway obstruction had declined by 24 hr following NGF treatment and was not significantly different from that of the PBS exposed group, the mean Penh difference was 1.10 ($P=0.22$, Figure 1B).

Effect of K252a on NGF enhanced citric acid-induced cough and airway obstruction

Studies have shown that the hyperalgesic effect of NGF is mediated via TrkA receptors. Therefore, we assessed the involvement of the TrkA receptor in the NGF enhanced citric acid-induced cough and airway obstruction. Pretreatment of guinea pigs with K252a (n=9) induced a statistically significant inhibition of the NGF enhanced citric acid-induced cough compared to vehicle (n=8) pretreated guinea pigs, the mean difference was 5.86 ($P=0.046$, Figure 2A). Moreover, pretreatment with K252a resulted in a statistically significant reduction in the NGF enhanced citric acid-induced airway obstruction compared to vehicle pretreated animals, the mean difference was 1.47 ($P=0.021$, Figure 2B).

Effect of IRTX on NGF enhanced citric acid-induced cough and airway obstruction
The involvement of TRPV1 in mediating the effects of NGF enhanced citric acid-induced cough and airway obstruction was investigated. Pretreatment of guinea pigs with IRTX (n=9) induced a statistically significant reduction in the NGF enhanced citric acid-induced cough compared to vehicle pretreated (n=8) guinea pigs, the mean difference was 15.68 (P=0.007, Figure 3A). Moreover, pretreatment with IRTX resulted in a statistically significant inhibition of the NGF enhanced citric acid-induced airway obstruction compared to vehicle pretreated animals, the mean difference was 3.37 (P=0.049, Figure 3B).

Effect of NGF and pretreatment with SB203580 on p38 MAPK levels in the lungs

To assess if the link between the activation of TrkA receptors and that of TRPV1 receptors is mediated via a p38 MAPK - dependent pathway, its phosphorylation following NGF treatment was investigated. Using a phospho-specific p38 antibody, we were able to detect basal expression of p38 MAPK in the lungs of control animals; BSA treated guinea pigs, (Figure 4A and B). NGF treatment induced a modest but statistically significant elevation in the levels of p38 MAPK phosphorylation in the lungs (P<0.001). Moreover, the NGF-induced p38 MAPK phosphorylation was prevented following pretreatment with SB203580 (P<0.001; Figure 4A and B).

Effect of SB203580 on NGF enhanced citric acid-induced cough and airway obstruction
These experiments were conducted to investigate if the MAPK inhibitor, SB203580, has an effect on the NGF enhanced citric acid-induced cough and airway obstruction. Pretreatment of guinea pigs with SB203580 (22.6 µg/ml; n=9) did not significantly affect the NGF enhanced citric acid-induced cough when compared to vehicle pretreated (n=8) guinea pigs, the mean difference was 0.85 (P=0.83, Figure 5A). Also, pretreatment with SB203580 did not significantly affect the NGF enhanced citric acid-induced airway obstruction compared to vehicle pretreated animals, the mean difference was 1.19 (P=0.27, Figure 5B).

DISCUSSION

The findings of this study show that NGF is not a tussigenic mediator. However, pre-exposure of guinea pigs to NGF significantly increases both the citric acid-induced cough and airway obstruction. This effect is seen within minutes of NGF exposure but was completely resolved after 24 hr. Additionally, the data show that NGF-induced enhancement of cough and airway obstruction is mediated predominantly via the TrkA receptor as treatment with K252a significantly reduced both these effects. Furthermore, pretreatment with IRTX also significantly reduced both the NGF enhancement of cough and airway obstruction suggesting an important role for TRPV1 in mediating the NGF enhancement of airway responses. Our data also show that NGF increases the phosphorylation of p38 MAPK in the lungs and this effect was significantly inhibited by the p38 MAPK inhibitor, SB203580. However, SB203580 did not inhibit the NGF mediated enhancement of either cough or airway obstruction suggesting that whilst p38 MAPK - dependent pathway is activated in the airways by NGF, it is not critical for the enhancement of these airway responses.

We selected the dose of 0.2 M citric acid on the basis of previous observations that this dose is a low grade stimulus for both cough and airway obstruction [24]. It was considered important to use a low dose of citric acid so that any NGF enhancment effects, on either cough or airway obstruction, would be more easily discernable. Additionally, as some studies have reported that IRTX can inhibit citric acid-induced baseline cough [4], it was important to ensure that any TRPV1 involvement was mainly NGF dependent.

Exposure of guinea pigs to NGF (0.3 and 1 μg /ml) had no direct effect on the cough reflex nor did NGF induce a statistically significant airway obstruction. Although it is possible that higher doses may have induced a cough response, this is unlikely as the 1 μg/ml dose induced some degree of airway obstruction in some animals yet those animals did not cough. Our data also show that NGF enhances the cough response to citric acid challenge. To the best of our knowledge this is the first report showing that NGF sensitizes the cough reflex. This effect was evident with 1 μg/ml but not the lower dose (0.3 μg/ml) and had disappeared by 24 hr suggesting that an acute exposure to NGF induces only an acute effect. This effect of NGF on the cough reflex is in agreement with findings in models of pain which demonstrate an important role for NGF in hyperalgesia. For example, both animal and human studies have shown that administration of NGF causes a robust mechanical and thermal hyperalgesia following either local or systemic administration [25-27]. Moreover, the time frame for the development of NGF-induced hyperalgesic effects was similar to what we have observed in the present study i.e. less than 30 minutes [25, 26]. Interestingly however, whereas chronic cough appears to be associated with inflammation, neurotrophins, including NGF, do not appear to be elevated [20]. However, a strong correlation between sputum NGF levels and the duration of cough exists [20]. Therefore, the role of NGF in both chronic human and acute cough associated with URTI still remains to be elucidated.

Our results show that airway obstruction to citric acid was also significantly enhanced following NGF pretreatment. This finding is in agreement with previous data showing that NGF enhances guinea pig tracheal strips contractile responses to histamine and also *in vivo* airway obstruction to both histamine and NKA [28-30]. The mechanism by which NGF may mediate this effect is postulated to be via both enhancement of neuropeptide release

and induction of their synthesis [11, 28, 29] and also possibly via a histamine receptor-dependent pathway [30].

The biological activity of NGF is mediated by two receptors: the high affinity TrkA receptor and the low affinity p75 NTR receptor [8]. In contrast to the p75 NTR which binds to all neurotrophins such as NGF, brain derived neurotrophin (BDNF) and neurotrophin-3/4/5 (NT-3/4/5) with equal affinities, the TrkA receptor is more selective for NGF [14, 31]. Because of the higher selectivity of the TrkA receptor for the NGF and the fact that this receptor has been associated with the effects of NGF in hyperalgesia and inflammation [19, 32], we investigated the role of TrkA receptors in mediating the NGF enhancement of cough and airway obstruction. Our data show that TrkA receptors play a central role in mediating the NGF- induced sensitization of airway effects as inhibition of this receptor with K252a significantly reduced both the NGF enhanced cough and airway obstruction. This is in agreement with studies showing that either neutralizing TrkA receptors with monoclonocal antibodies or inhibiting them with the K252a inhibits the NGF induced hyperalgesia [33, 34]. Therefore, our data are a further confirmation of the importance of this receptor in sensitizing sensory nerves. However, recent evidence has also suggested a role for the p75 NTR in NGF mediated sensitization of sensory nerves [35] and hence a role of this receptor in these airway responses remains to be established.

The heat – gated ion channel TRPV1 is an important detector of noxious levels of heat and evidence is accumulating to suggest that this receptor appears to play a critical role in hyperalgesia [36]. Hence, the role of TRPV1 in mediating the NGF enhanced cough and airway obstruction was investigated. Interestingly, we observed that the TRPV1 antagonist, IRTX, very effectively inhibited both the NGF enhanced cough and airway obstruction. Although it has been previously reported that TRPV1 are partly implicated in the baseline citric acid-induced cough [4], baseline cough was negligible in our study and hence it can be strongly argued that the IRTX effects are due to the inhibition of the NGF enhancement of airway effects. This finding also fits in with data obtained in several studies. For example, in culture, NGF rapidly potentiates the activity of TRPV1 channels in DRG neurons treated with capsaicin [37]. Zhang and co-workers [19] have also reported that NGF enhances TRPV1 function, which was associated with TRPV1 phosphorylation, and also their increased membrane expression. Furthermore, a recent study has provided convincing data showing that NGF sensitizes TRPV1 mediated signaling and that this was not via an increase in the unitary conductance or activation of quiescent channels but rather through an increase in the total number of plasma membrane TRPV1 channels [38]. It is also noteworthy to mention that these NGF-induced effects in both studies were observed within minutes i.e. a rapid effect which is in accordance with the time frame of the responses that were noted in our experiments.

In addition to confirming the involvement of TRPV1 in the NGF enhancement of cough and airway obstruction, we investigated the possible biochemical pathway linking TrkA and TRPV1 receptors which thus far remains controversial with several candidate signaling pathways proposed [18, 19, 38-40].

TrkA receptor signaling is currently thought to be mediated by three signaling pathways which include p38 MAPK, PI3K and PLCγ [18, 39, 40]. Our data show that basal levels of phosphorylated p38 MAPK are indeed modestly increased following NGF treatment. This evidence would suggest that the NGF–TrkA signaling is, at least partly, mediated via the p38 MAPK – dependent pathway. Moreover, the p38 MAPK inhibitor, SB203580, significantly reduced the NGF-induced p38 MAPK phosphorylation. Surprisingly however, our data showed that SB203580 did not affect the NGF enhanced cough or airway obstruction. This suggests that the p38 MAPK - dependent pathway, although activated by NGF, does not seem to be involved in enhancing cough or airway obstruction to NGF. An alternative paradigm would be via the PI3K and PLCγ pathways. Indeed, evidence exists showing that these signaling pathways are activated by NGF and can enhance TRPV1 expression [38-40]. Hence, the involvement of these pathways in NGF enhanced cough and airway obstruction needs to be investigated. It is also important to mention that several other mediators such as PGE_2 and histamine [30, 41, 42] have been implicated in the signaling of NGF mediated airway responses and hence their involvement in the enhancement of cough and airway obstruction cannot be ruled out. In our study, the location of the TrkA receptors was not addressed; however they are known to be expressed in non-neuronal as well as neuronal sites [43-46]. Also, a central site of action for NGF in enhancing cough is likely and remains to be determined.

In summary, our data show that although NGF does not directly induce cough or significant airway obstruction, it significantly enhances both the citric acid- induced cough and airway obstruction. These effects are mediated via both the TrkA and TRPV1 receptors and although NGF activates p38 MAPK in the lungs, this pathway does not appear to be involved in mediating the NGF enhancement of cough or airway obstruction. Due to the possible involvement of the TRPV1 in baseline cough; our findings would suggest that the TrkA receptor may be an attractive drug target for inhibiting enhanced cough without affecting the normal cough reflex.

ACKNOWLEDGEMENTS

We are very grateful to Professor Saghir Akhtar for doing the western blot experiments. We would like to thank Dr Abdelmoneim Awad and Prof Yunus Luqmani for their constructive comments on the manuscript and also Heba Abdou for her initial technical work on this project. We also acknowledge the support of Ola Zahran and Sunny Okojo from the animal resource center.

FOOTNOTES

Additional Methods data are published online only.

Funding: This study was funded by Kuwait University Research Administration grant number PT01/05

Competing interests: The authors declare that they have no competing interests

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Ethical approval: The study was approved by the Animal Welfare and Use of Laboratory Animals' Committee at the Health Sciences Center, Kuwait University

Figure legends

Figure 1. Effect of NGF treatment both immediately (0.3 and 1 µg/ml) and post 24 hr (1 µg/ml) on citric acid-induced cough (A) and airway obstruction (B) in guinea pigs. Values are presented as mean+SEM. * Statistically significant difference (P<0.05) compared to PBS, BSA, 0.3 µg/ml (immediate) and 1 µg/ml (post 24 hr) NGF treated animals.

Figure 2. Effect of K252a (20 µg/kg; i.p.) on NGF enhanced citric acid-induced cough (A) and airway obstruction (B) in guinea pigs. Values are presented as mean+SEM. *Statistically significant difference (P<0.05) compared to vehicle treated animals.

Figure 3. Effect of IRTX (7.5 µg/ml) on NGF enhanced citric acid-induced cough (A) and airway obstruction (B) in guinea pigs. Values are presented as mean+SEM. * Statistically significant difference (P<0.05) compared to vehicle treated animals.

Figure 4. (A) Western blotting analysis of phosphorylated p38 MAPK protein levels from lungs of guinea pigs exposed to BSA (control), vehicle or SB203580 pretreated guinea pigs and exposed to NGF (B) Histogram of western blots showing the NGF-induced elevation in p38 MAPK phosphorylation and subsequent reduction after SB203580 pretreatment. Data shown represents mean+SEM of p38 phosphorylation intensity relative to actin (n=3). * Statistically significant difference (P<0.05) compared to BSA exposed animals. ** Statistically significant difference (P<0.05) compared to vehicle treated animals.

Figure 5. Effect of SB203580 (22.6 µg/ml) on NGF enhanced citric acid-induced cough (A) and airway obstruction (B) in guinea pigs. Values are presented as mean+SEM.

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Measurement of airway obstruction

Conscious unrestrained guinea pigs were placed individually in a transparent whole body plethysmograph box (Buxco, Troy, NY). A pneumotachograph, with defined resistance in the wall of the main chamber, acted as a low-pass filter and allowed thermal compensation. The chamber was fitted with a microphone and connected to both an external speaker and a computer to allow visualization of the sound signal. The plethysmograph was also connected to a bias flow regulator that was supplying air at a rate of 3 l/min and withdrawing air at a rate of 4 l/min; the difference being taken up by airflow into the box through the pneumotachograph. Briefly, the pressure differences between the main chamber of the whole body plethysmograph containing the animals and a reference chamber (box pressure signal) were measured. Depending on these box pressure signals, the phases of the respiratory cycle, peak inspiratory pressure (PIP), peak expiratory pressure (PEP), and tidal volumes, and an index of airway caliber, enhanced pause, (Penh) was calculated as follows:

$$Penh = Pause \times \frac{PEP}{PIP}$$

Penh is a dimensionless value that reflects changes in the waveform of the box pressure signal from inspiration and expiration (PIP, PEP) and combines it with the timing comparison of early and late expiration (Pause).

The animals were exposed to nebulized solutions containing various agents. Aerosols were generated using a DeVilbiss aerogen ultrasonic nebulizer (DeVilbiss, Somerset, PA, USA) and had an aerodynamic mass median diameter range of 1-5 μm (manufacturer's indication). The animals were allowed a settling period in the whole body plethysmograph, after which baseline airway function was recorded for 2 min before starting aerosol. Animals in all groups were then exposed to citric acid (0.2 M) aerosol for 10 min during which cough and airway function were recorded and for 5 min thereafter (total 15 min). All drugs or vehicles given by inhalation were also aerosolized using the aerogen ultrasonic nebulizer over a 10 min period. Assessment of cough and airway obstruction were performed simultaneously.

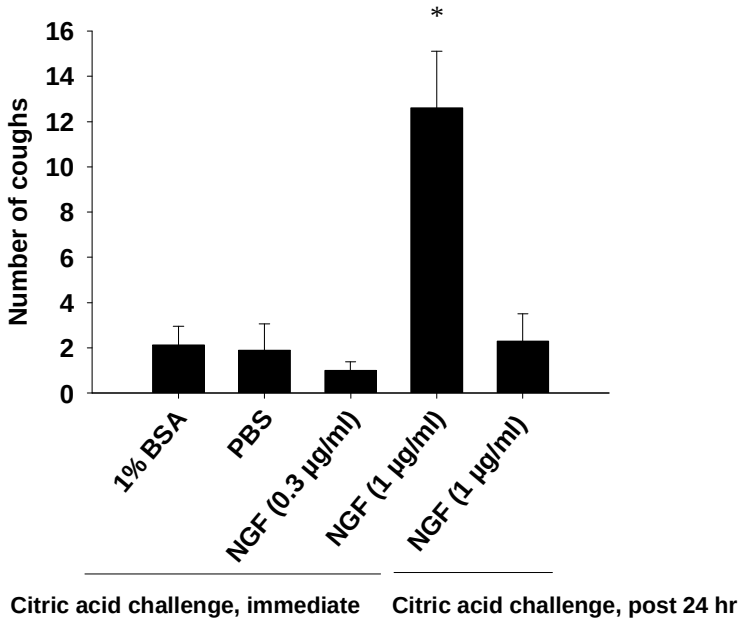
Protein isolation from the lungs

After completion of the cough and airway obstruction experiments, the animals were sacrificed by cervical dislocation. Both lungs were removed, washed with phosphate buffered saline (pH 7.4) at 4 º C and snap frozen in liquid nitrogen followed by storage at -80 º C. The samples were freeze-thawed, homogenized, sonicated, and separated by density centrifugation. Supernatants were then analyzed for total protein.

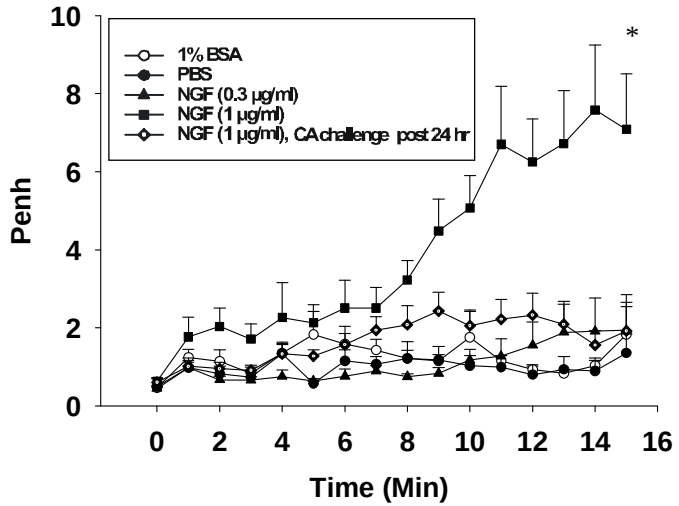
Western blot analysis

Equal amounts of protein (50 μg/lane) were electrophoresed on SDS-PAGE (5% stacking gel and 8% resolving gel) and blotted onto nitrocellulose membranes. The membranes were blocked with 2% non-fat milk solution in PBS and incubated with the indicated primary antibodies (at 1/1000 dilution for 1 hr), then with horseradish peroxidase conjugated secondary antibodies for 30 min. β-actin was used as loading control for western blot analysis. The membranes were then washed 3 times with PBS and signals were visualized using the ECL kit according to the manufacturer's instructions. Immunoreactive bands were detected using autoradiography film. Quantification and measurement of the bands were performed with a densitometer (Biorad, USA).

1 A



1 B



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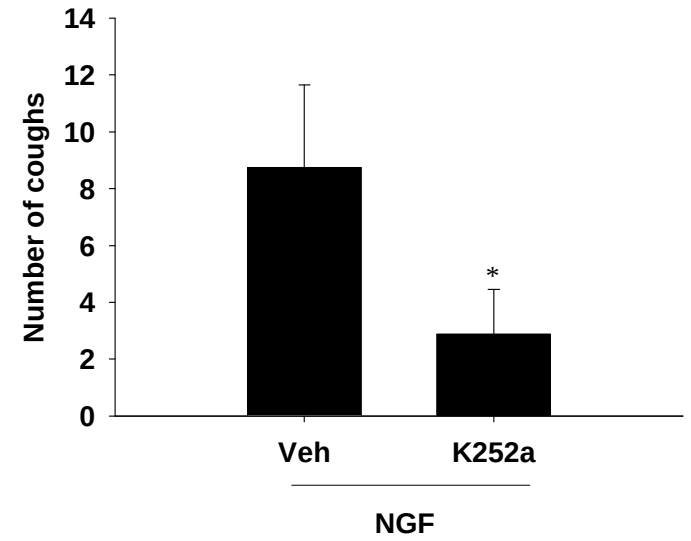

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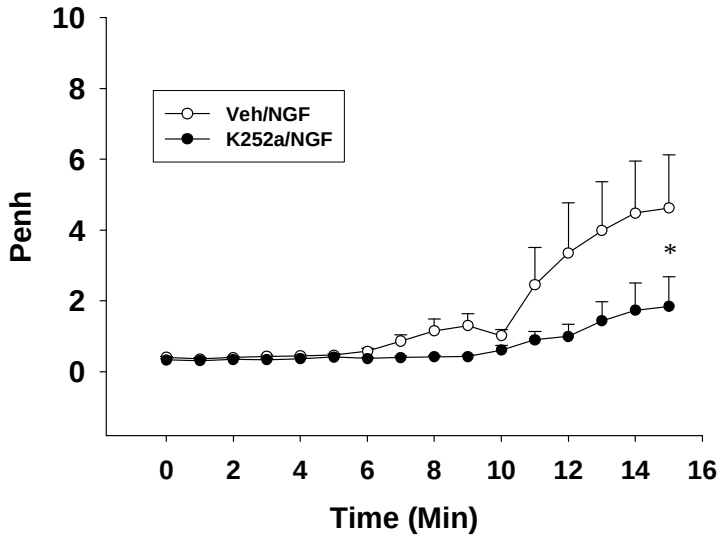

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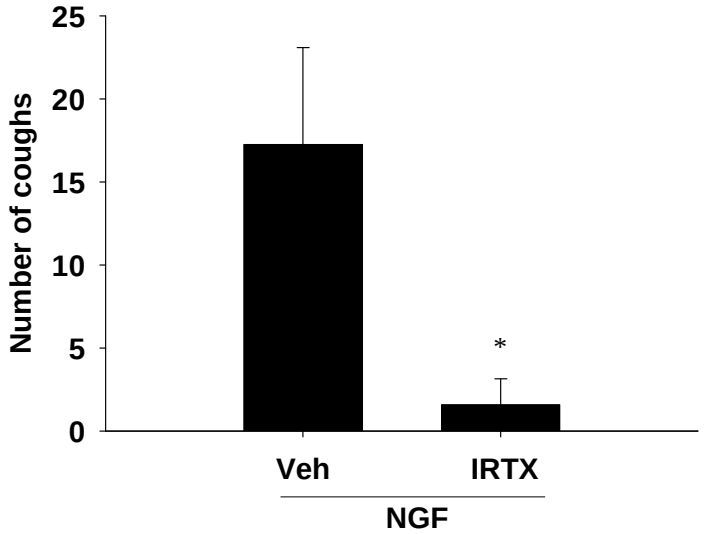

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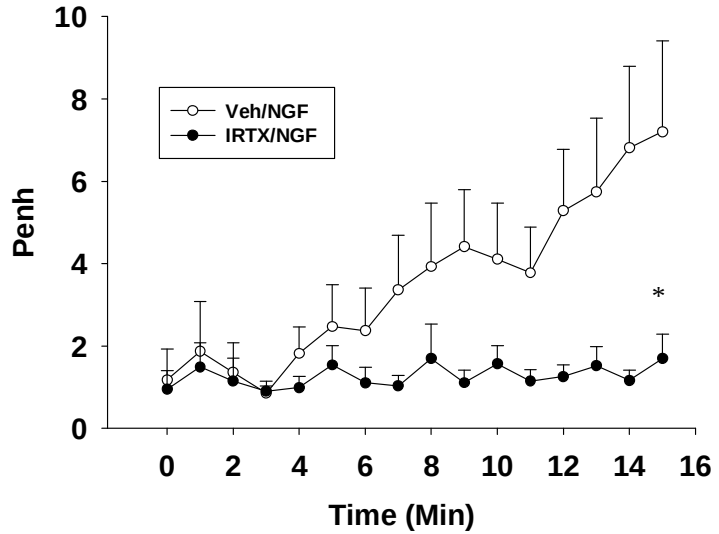

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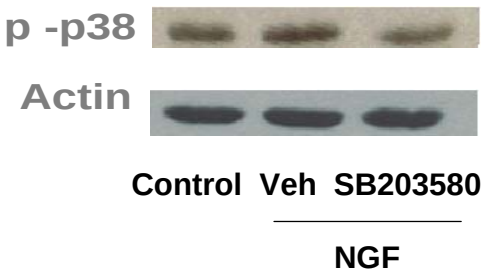

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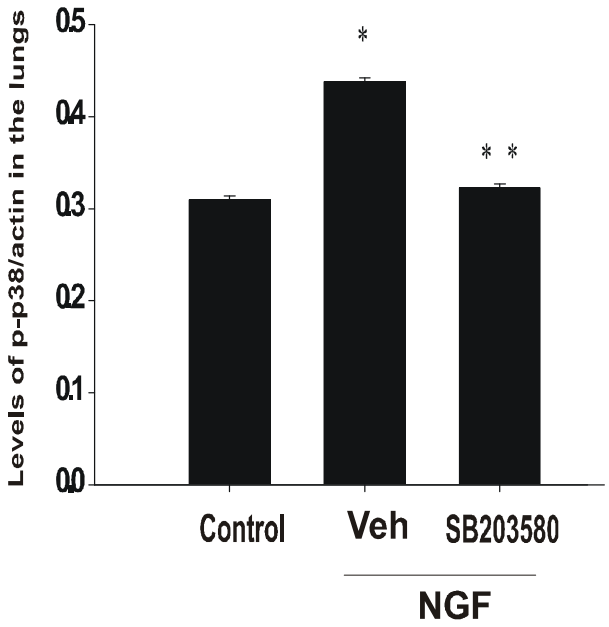
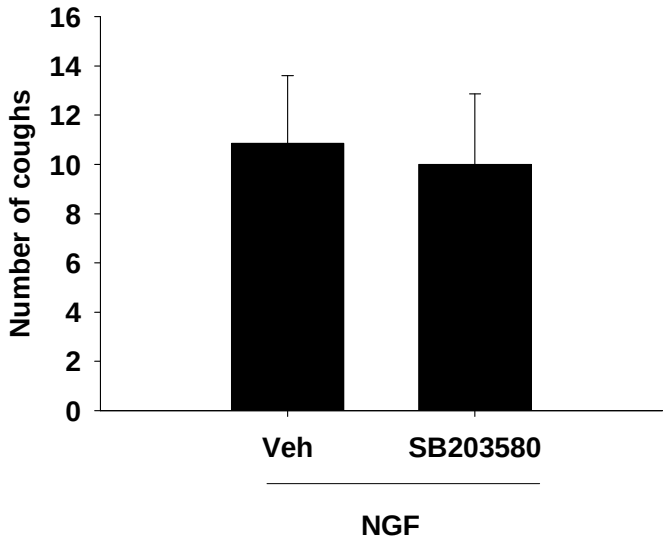


5 A



5 B