Mechanism of bronchoprotective effects of a novel natriuretic hormone peptide

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Background: The natriuretic hormone peptide (NHP) $_{99\text{-}126}$, a C-terminal peptide of pro-atrial natriuretic factor (proANF), induces bronchodilatory effects in people with asthma. Recently, another plasmid-encoded C-terminal peptide, $pNHP_{73\text{-}102}$, was shown to induce a long-lasting bronchoprotective effect in a mouse model of allergic asthma.

Objective: This study was carried out to determine the role of lung epithelial cells in the bronchoprotective and anti-inflammatory activity of these peptides.

Methods: Human type II alveolar epithelial cells (A549) and normal human bronchial epithelial (NHBE) cells were transfected with $pNHP_{73\text{-}102}$ to test the effect of this peptide on activation of these cells. After transfection, cells were analyzed for changes in Ca^{++} and nitric oxide (NO) levels. Also, activation of NFκB and the extracellularly regulated kinase (ERK) 1, 2 signaling pathway was examined by luciferase reporter assay and phosphorylation studies respectively.

Results: Analysis of intracellular Ca^{++} levels in $pNHP_{73\text{-}102}$ –transfected A549 or NHBE showed that the peptide increases release. This Ca^{++} release was accompanied by an increase in the production of NO. Also, overexpression of $pNHP_{73\text{-}102}$, but not pVAX control, in phorbol myristate acetate–activated A549 cells resulted in a significant decrease in expression of a cotransfected nuclear factorκB (NFκB)–luciferase reporter. Similarly, $pNHP_{73\text{-}102}$ decreased TNF-α–induced NFκB activation in NHBE cells. Furthermore, $NHP_{73\text{-}102}$ but not atrial natriuretic peptide decreased phosphorylation of Erk-1, 2 in A549 cells.

Conclusions: Overexpression of $pNHP_{73\text{-}102}$ in epithelial cells causes increased production of intracellular Ca^{++} and NO, with a concomitant decrease in activation of NFκB and ERK1, 2. These results suggest a bronchodilatory and anti-inflammatory activity of this peptide. (J Allergy Clin Immunol 2004;113:79-85.)

Key words: *ANP, asthma, NFκB, ERK, anti-inflammatory, bronchodilation, natriuretic, signaling*

Abbreviations used

ANP: Atrial natriuretic peptide
cGMP: Cyclic GMP
cNOS: Constitutive form of nitric oxide synthase
ERK: Extracellularly regulated kinase
NFκB: Nuclear factor kappa B
NHBE: Normal human bronchial epithelial (cells)
NHP: Natriuretic hormone peptide
NO: Nitric oxide
NPR: Natriuretic peptide receptor
UD: Urodilatin

Airway epithelial cells play important roles in modulating inflammatory response to allergens, viruses, and pollutants and seem to be involved in bronchoconstriction and airway inflammation, which are the hallmarks of asthma. In addition to the incumbent environmental trigger, a complex array of cellular endocrine, metabolic, and physiologic activities contributes to the dynamic immunologic processes leading to the development of immunity and inflammation. In particular, a family of natriuretic hormone peptides (NHP) have been described that play a significant role in the lung and that include the 4 peptides generated from atrial natriuretic factor (ANF), including the C-terminal peptide comprising residues 99-126, called ANP (now designated as $NHP_{99\text{-}126}$). Three isoforms of ANP, brain natriuretic peptide, C-type natriuretic peptide, and urodilatin, have been reported.[1] Lung cells express the guanylyl cyclase–coupled natriuretic peptide receptor (NPR) A and the guanylyl cyclase–uncoupled receptor NPR-C.[2] ANP is thought to signal primarily through NPR-A that increases cyclic GMP (cGMP) and activates cGMP-dependent protein kinase. NPR-C functions as a clearance receptor but also seems to signal phospholipase C activation and a decrease in adenylyl cyclase activity.[3-5]

The ANP level is significantly increased during severe asthma attacks, suggesting that ANP might have important bronchoprotective effects in asthma.[6] ANP induces relaxation of pulmonary arteries and dilates the trachea and bronchi in humans and various animal species.[7-12] In ovalbumin-induced asthmatic guinea pigs, intravenous ANP inhibits antigen-induced bronchoconstriction.[13] In humans, exogenous ANP reverses airway hyperreactivity when given intravenously or by inhalation[14,15] and has also been shown to modify bronchial reactivity to inhaled

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histamine,[16] propanolol,[17] and nebulized water.[18] Despite being similar to albuterol in its bronchodilating ability, the short half-life in vivo of ANP peptides (3 to 5 minutes) has limited their usefulness in the treatment of asthma.[19]

Previously, we reported that overexpression in the lung of a plasmid-encoded ANF peptide, NHP_{73-102}, led to a significant reduction of airway reactivity lasting up to 3 weeks in a mouse model of ovalbumin-induced asthma.[20] Mice receiving $pNHP_{73-102}$ showed significantly decreased airway hyperreactivity to methacholine challenge compared with mice receiving vector alone.[20] Given the central role of epithelial cells in asthma pathology and because ANP and NPR-A have been identified in type II alveolar epithelial cells of rodents,[21,22] we reasoned that epithelial cells might shed light on the mechanism of NHP_{73-102}–induced bronchodilation. To test this hypothesis, type II alveolar epithelial cells (A549) and normal human bronchial epithelial (NHBE) cells were examined after transfection with $pNHP_{73-102}$. The results show that de novo NHP_{73-102} expression in lung epithelial cells results in increased production of nitric oxide (NO) and decreased activation of signaling pathways involved in airway inflammation.

METHODS

Cell lines and culture conditions

The human alveolar type II epithelial cell line A549 (ATCC) was cultured at 37°C in Dulbecco modified Eagle medium containing 10% FBS and 100 U/mL each of penicillin and streptomycin in an atmosphere of 5% CO_2/95% air. Cells were subcultured weekly and used between passages 9 and 22. Experiments were repeated with primary human bronchial epithelial cells (NHBE) obtained from Clonetics (Walkersville, Md) from pooled donors. These cells were cultured in Bronchial Epithelial Basal Medium (Cambrex Bio Science, Walkersville, Md) medium supplied by the vendor and supplemented with 10% FBS and a mix of growth factors without antibiotics. Cells were grown at 37°C in 5% CO_2/95% and used between passages 3 and 9.

Expression plasmids and transfection

The construction of the plasmid encoding NHP_{73-102} has been described previously.[20] For transfection of epithelial cells, cells at 60% confluence (log phase) were transfected 4 hours at 37°C with plasmid DNA (1 μg/10^6 cells) complexed with lipofectamine (GibcoBRL Life Technologies, Carlsbad, Calif). Complete medium was then added to the cultures, and the cells were incubated at 37°C for 24 to 48 hours to allow expression of the natriuretic peptides.

Assay for intracellular calcium

Cells grown in 12-well tissue culture plates were transfected with NHP_{73-102} or control vector (pVAX) and allowed to express the gene product for various times at 37°C. Relative intracellular calcium levels were measured by use of the fura-2 dual excitation ratio method.[23] Growth medium was removed from transfected cells and replaced with PBS containing the cell-permeable acetoxymethyl ester of fura-2 (Molecular Probes, Eugene, Ore), and loading of fura-2-AM was allowed to continue for 1 hour at 37°C. Cells were washed with PBS, and plates were read in a Dynex MRX fluorescence plate reader (Dynatech, Chantilly, Va) with dual excitation at 330 nm and 380 nm and emission at 515 nm. Data are represented as relative change in 330/380 ratio for experimental samples compared with vector controls.

Assay for NO

The assay for NO is based on that of Misko et al[24] and measures nitrite, the stable breakdown product of NO, which is reacted with diaminonaphthalene to produce a fluorescent compound. A549 or NHBE cells were transfected with plasmid/lipofectamine complexes as described previously. At specific time points, 100-μL samples of culture medium were removed and stored at –20°C. After all samples were taken, they were cleared by centrifugation, and 10 μL of a freshly prepared solution of 0.02 mg/mL diaminonaphthalene was added to each tube, shaken, and allowed to react for 10 minutes at room temperature. The reaction was stopped by addition of 30 μL 0.5 mol/L NaOH, and the fluorescence of the samples was read with a quartz microcuvet (3-mm path length) in a Jasco spectrofluorometer (Jasco, Easton, Md), with excitation at 365 nm and emission at 409 nm. Nitrite standards were run in the same medium as the experimental samples to generate a standard curve that was used to calibrate the readings. As a positive control, 1 set of wells was incubated with 1 μmol/L calcium ionophore, A23187 (Sigma).

Nuclear factor kappa B (NFκB) luciferase reporter assay

A549 or NHBE cells were co-transfected with NHP_{73-102} or vector alone (pVAX), reporter plasmid (pNFκB-Luc, Mercury Profiling System, Clontech, Palo Alto, Calif), a transfection normalization vector (pLacZ), and a negative control vector (pTA-Luc). DNA (0.5-1 μg/10^6 cells) was transfected into 60% confluent A549 and NHBE cells by use of lipofectamine (Life Technologies) for 4 hours. As a positive control, NFκB was activated 24 hours after transfection with 20 ng/mL PMA (A549) or 100 U/mL TNF-a (NHBE). Luciferase activity was detected with the Luciferase Reporter Assay kit (BD Biosciences/Clontech) and a Dynex luminometer (Dynatech). Data (n = 3) are expressed as fold change relative to vector control after subtraction of background and normalization.

Immunoblot analysis

A549 cells transfected with NHP_{73-102} or control as described previously were lysed 24 hours after transfection, and total cellular protein (50 μg) was separated by SDS-PAGE, blotted to nitrocellulose, and incubated with antibodies to specific proteins (Santa Cruz, Santa Cruz, Calif). Bands were visualized by enhanced chemiluminescence (Amersham Life Sciences, Piscataway, NJ) on Kodak X-OMAT-AR film.

Statistical analysis

Experiments were repeated a minimum of 3 times, and data are expressed as means ± SEM. Pairs of groups were compared through the use of Student *t* tests. Differences between groups were considered significant at $P \leq .05$.

RESULTS

NHP_{73-102} modulates intracellular calcium

A diagram of the peptide used in this study and other natriuretic peptides is shown in Fig 1. The prohormone is cleaved into 3 N-terminal peptides, each having a specific function, plus the C-terminal ANP. The peptide used in this study includes 4 residues of ANP and 8 of urodilatin. Because ANP has been shown to increase Ca^{++} in endothelial cells,[25] we investigated whether NHP_{73-102} could alter Ca^{++} levels in A549 or NHBE epithelial cells. Cells transfected with control vector or NHP_{73-102} were examined at the indicated times after transfection for

intracellular Ca^{++} by use of fura-2-AM. Ca^{++} was elevated in the cells transfected with NHP_{73-102} compared with controls (Fig 2).

NHP_{73-102} increases NO production from epithelial cells

NO is a bronchodilator, and Ca^{++}-calmodulin binding activates the constitutive form of nitric oxide synthase (cNOS) in epithelial cells.[26] To determine whether the increased intracellular Ca^{++} seen in NHP_{73-102}–transfected cells affects NO levels, aliquots of the medium were removed before the Ca^{++} assay and mixed with diaminonaphthalene, which reacts with nitrite (from the reaction of NO and water) to produce a fluorescent compound. NO generation was significantly higher in cells expressing NHP_{73-102} (Fig 3, *A* and *B*). To verify that NO production was due to the constitutive NOS, 1 aliquot of cells was incubated during the expression phase with 1 mmol/L N_{ω}-nitro-L-arginine methyl ester, an arginine analog that blocks cNOS production of NO. The enhanced NO generation was inhibited by pretreatment of the cells with N_{ω}-nitro-L-arginine methyl ester, which blocks cNOS activity (Fig 3, *C*).

NHP_{73-102} decreases NFκB activation

Bronchial hyperreactivity**,** one of the hallmarks of asthma, might be caused by airway inflammation, and the transcription factor NFκB is a central player in inflammation of the airway epithelium.[27] To determine whether NHP_{73-102} plays a role in modulating inflammatory mediators produced by epithelial cells, the effects of overexpression of NHP_{73-102} in human lung epithelial cells on activation of NFκB were examined. A549 (Fig 4, *A*) or NHBE (Fig 4, *B*) cells were co-transfected with $pNHP_{73-102}$ and a luciferase reporter plasmid containing an upstream consensus sequence for NFκB binding. NFκB was activated by treatment of the cells with phorbol 12-myristate 13-acetate (A549) or TNF-α (NHBE). NHP_{73-102} significantly decreased luciferase expression in cells cotransfected with $pNHP_{73-102}$ compared with control plasmid (Fig 4, *A* and *B*). NFκB is inactivated by binding to a repressor protein, IκB, which prevents it from translocating to the nucleus. Western blots of cell lysates for IκB revealed that NHP_{73-102} and ANP increased levels of IκB relative to the vector control Fig 4, *C*).

NHP_{73-102} represses phosphorylation of extracellularly regulated kinase (ERK)-1, 2

The second commonly used pathway in inflammatory diseases such as asthma involves ERKs, especially ERK-1, 2, which are activated through a protein kinase cascade. Because ANP has been shown to increase ERK activity in human endothelial cells,[28,29] the effect of NHP_{73-102} on ERK-1, 2 phosphorylation was examined in A549 cells. The results show that NHP_{73-102} decreased ERK-1, 2 phosphorylation, whereas the expression of C-terminal NHP_{99-126} did not affect phorbol 12-myristate 13-acetate–activated ERK-1, 2 (Fig 5).

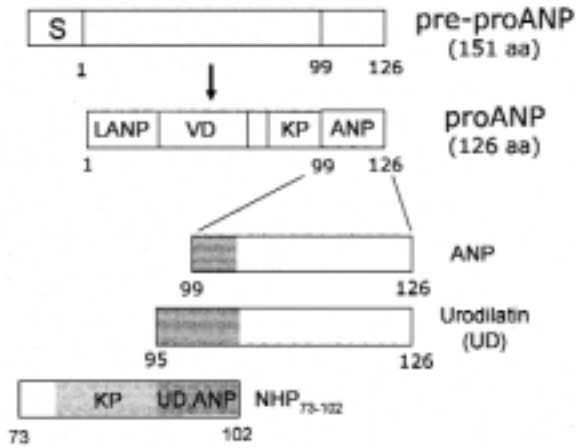

FIG 1. Diagram depicting the family of natriuretic hormone peptides. Translation of the atrial natriuretic factor gene results in a pre-prohormone that is processed into a prohormone, which is then cleaved into several bioactive peptides: long-acting natriuretic peptide (LANP), vessel dilator (VD), kaliuretic peptide (KP), and atrial natriuretic peptide (ANP). Urodilatin (UD) is a variant of ANP that has been implicated in asthma. The natriuretic hormone peptide construct used in this study, NHP_{73-102}, is distinct from the preceding peptides but includes an overlap region shared with UD.

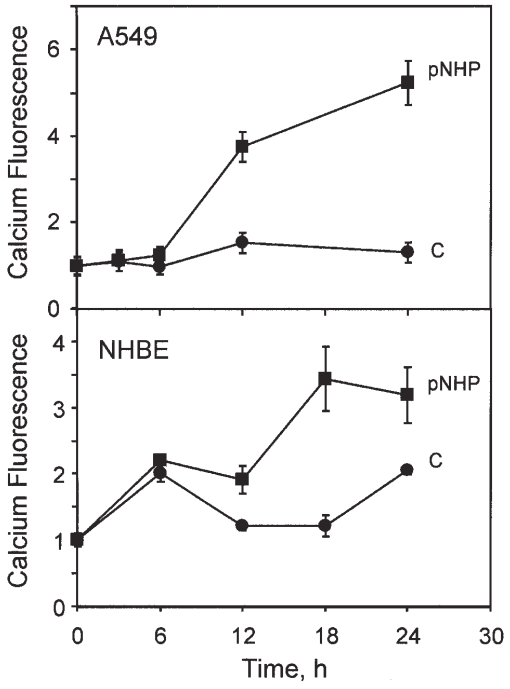

FIG 2. Overexpression of natriuretic hormone peptide $(NHP)_{73-102}$ leads to increased levels of intracellular calcium. Human type II alveolar epithelial cells (A549) (*upper panel*) and normal human bronchial epithelial (NHBE) (*lower panel*) cells were transfected with control vector or NHP_{73-102}. At the indicated times after transfection, cells were incubated with 1 mmol/L fura-2 (Molecular Probes), and fluorescence was determined by the ratiometric method. Data are expressed as fold change relative to control cells at time zero and are the averages of 3 wells ± SEM (n = 3).

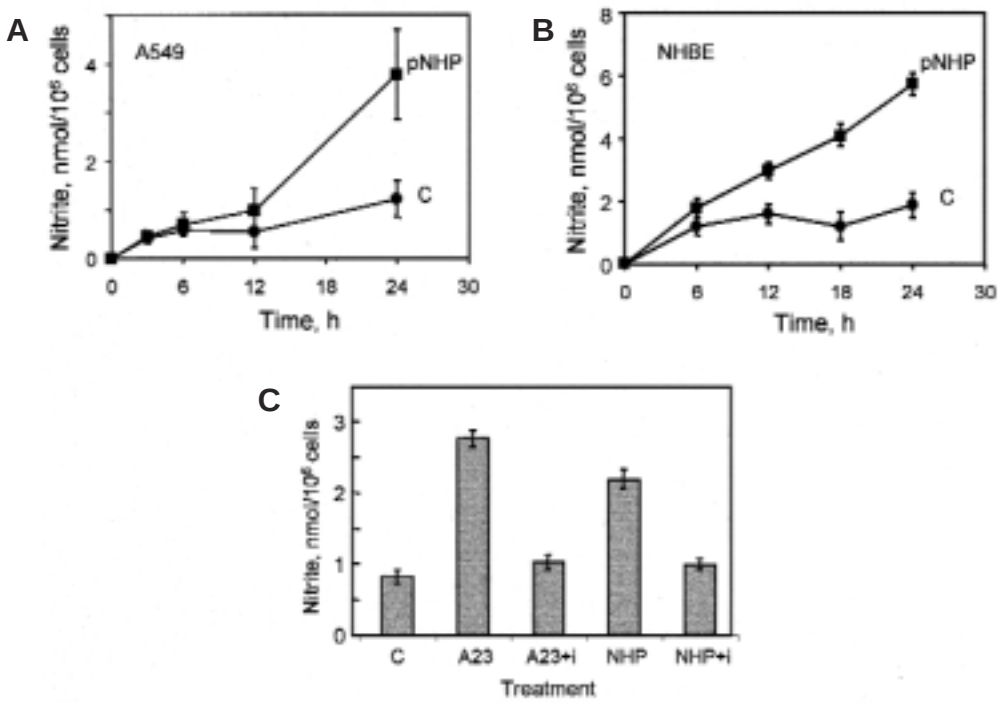

FIG 3. Overexpression of natriuretic hormone peptide $(NHP)_{73\text{-}102}$ leads to increased production of nitric oxide. Human type II alveolar epithelial cells (A549) **(A)** and normal human bronchial epithelial (NHBE) **(B)** cells were transfected with control vector or $NHP_{73\text{-}102}$. At the indicated times after transfection, aliquots of the culture medium were assayed for nitrite (the nitric oxide [NO] reaction product). Fluorescence was read at 409 nm with excitation at 365 nm by use of a Jasco spectrofluorometer. Data are means ± SEM (n = 3). **C,** A549 cells were pretreated with cNOS inhibitor *(i)*, N_{ω}-nitro-L-arginine methyl ester, or calcium ionophore *(A23)* before being incubated and assayed for NO.

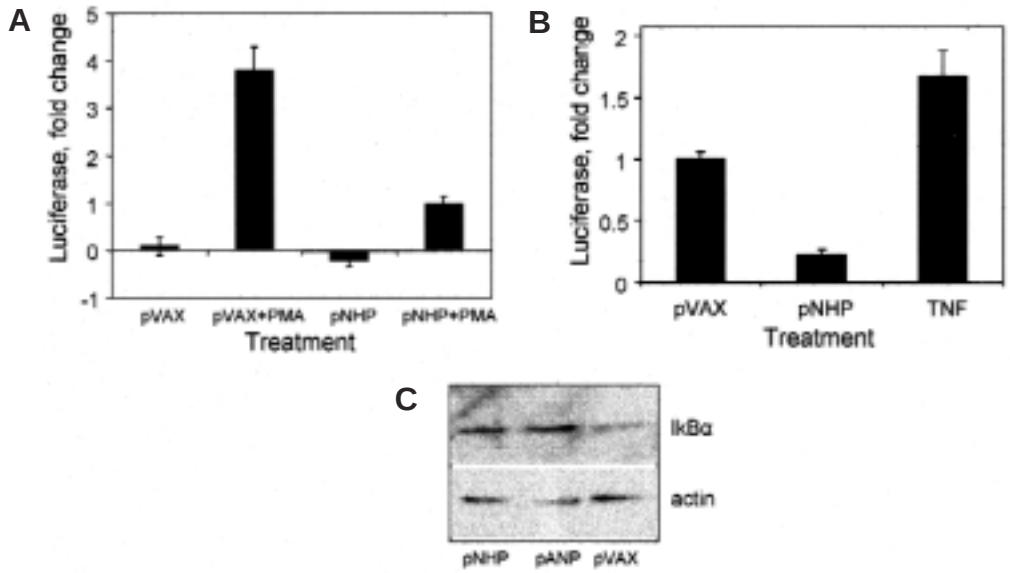

FIG 4. Natriuretic hormone peptide $(NHP)_{73\text{-}102}$ induces repression of NFκB-luciferase reporter gene. Human type II alveolar epithelial (A549) **(A)** or normal human bronchial epithelial (NHBE) **(B)** cells were co-transfected with $pNHP_{73\text{-}102}$ or vector (pVAX) alone, pNFκB-luc reporter plasmid, and pLacZ normalization control. NFκB was activated 24 hours after transfection by incubating cells with 20 ng/mL phorbol 12-myristate 13-acetate (A549) or 100 U/mL TNF (NHBE). Luciferase activity data (average of 3 readings) are expressed as fold change relative to vector control. To determine whether changes in NFκB activity correlated with activation of the NFκB repressor, IκBα, A549 cells were lysed 24 hours after transfection, and protein was immunoblotted for IκBα. Actin was used as a gel loading control **(C)**.

DISCUSSION

This report describes for the first time the mechanism underlying the action of a NHP, which combines both bronchodilatory and anti-inflammatory effects and has the potential of being a useful therapeutic agent in the treatment of airway diseases. This finding is significant, because the most effective treatment of asthma and allergic diseases might require the use of novel drugs or drug combinations that can produce both bronchodilation and reduce inflammation. β2-agonists are considered the first line of therapy for asthma because of their bronchodilatory property; however, their effects only last for a few hours, requiring frequent dosing. Corticosteroids are considered the "gold standard" for anti-inflammatory treatment in patients with asthma, and recent trends in developing more effective treatments combine both β2-agonists and steroids. However, desensitization to β2-agonists and potential adverse effects of high-dose or systemic steroids limit the long-term use of these drugs.

One of the important results of this study is that overexpression of NHP_{73-102} increases intracellular Ca^{++} concentration in epithelial cells. Changes in Ca^{++} are important, because they might affect bronchoconstriction directly in airway smooth muscle cells.[30] In guinea pig trachea, ANP (aa 99-126) was a more potent relaxant than C-type natriuretic peptide, whereas ANP (aa 4-23) was inactive, suggesting the involvement of NPR-A receptors in the relaxant effect of ANP.[10] Extracellular Ca^{++} has been reported to affect eosinophil migration,[31] IL-9 expression, and mucus production[32]; however, the role of intracellular Ca^{++} in airway epithelial cells has not been studied previously. Kiemer and Vollmar[33] reported that treatment of murine macrophages with ANP resulted in elevated intracellular calcium and inhibition of the inducible isoform of NOS. In contrast to epithelial cells in which the constitutive NOS is activated by intracellular Ca^{++} release, macrophages possess only inducible NOS, which can be repressed by Ca^{++} through destabilization of its mRNA.[34] The increased production of NO by NHP_{73-102} overexpression in airway epithelial cells reported here could be explained in terms of activation of the constitutive epithelial NOS by the increase in intracellular calcium.[35] The significance of our result is at present unclear, particularly in the context of asthma, but it may be physiologically relevant, because NO has been found to affect airway hyperreactivity in animal models and in human asthma.[36-38] Previous studies have reported evidence for antiproliferative effects of ANP and NO in human airway smooth muscle cells mediated through cGMP-dependent and cGMP-independent mechanisms.[39] Airway smooth muscle hypertrophy and hyperplasia are important determinants of airway remodeling and bronchial responsiveness in asthma. NHP_{73-102} seems to act on epithelial cells to produce NO by means of constitutive NOS, which in turn controls bronchial hyperreactivity and proliferation of airway smooth muscle cells. This differential activity of ANP has also been reported in congestive heart failure.[40]

Concomitant with the increased production of NO, NHP_{73-102} expression caused a decrease in activation of NFκB. This is a significant finding, because NFκB is a central mediator of airway inflammation in patients with asthma.[27] Although seen for the first time in airway epithelial cells, our results are consistent with previous observations that ANP mediates anti-inflammatory[41] and cytoprotective[42] effects. Inactivation of NFκB leads to a decrease in the cytokine-stimulated and stress-stimulated activation of various cell types, decreases pro-inflammatory cytokine production,[43] and can reduce TNF-α–stimulated production of adhesion molecules in endothelium.[44] It is likely therefore that decreased NFκB activation results in decreased pulmonary inflammation, which might lead to reduced asthma severity. The relatively greater inhibition of luciferase activity in A549 cells by NHP_{73-102} (Fig 4, *A*) compared with the NHP-mediated increase in IκB expression seen in the Western blot (Fig 4, *C*) might be explained by the fact that the A549 cells in the reporter gene experiments were treated with phorbol 12-myristate 13-acetate, whereas those for the Western blot were not. The phorbol 12-myristate 13-acetate–mediated inflammatory state might reflect more closely what occurs in asthma.

In addition to the effects on NFκB, the results showed that NHP_{73-102} also decreased ERK-1, 2 phosphorylation. This is distinct from the ANP-mediated attenuation of TNF-α–induced actin polymerization, through activation of MAPK phosphatase-1 and inhibition of p38 activity leading to decreased permeability.[45] Most recently, ANP has been shown to increase ERK activity in human endothelial cells.[28, 29] ERK-1, 2 is important, because it plays a role in activating the transcription factor, activator protein-1, which regulates the expression of genes involved in inflammation. The evidence that NHP_{73-102} decreases phorbol 12-myristate 13-acetate–induced phosphorylation of ERK-1, 2 compared with ANP indicates that NHP_{73-102} might possess antagonistic effects in terms of ERK-1, 2 activation.

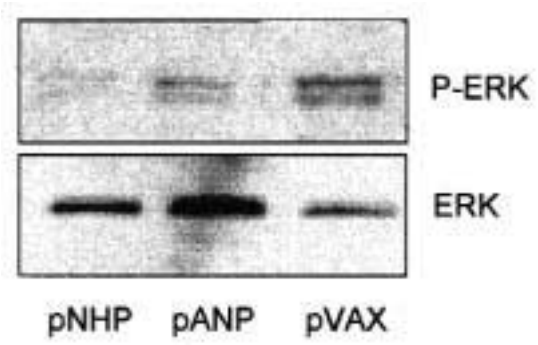

FIG 5. Overexpression of natriuretic hormone peptide $(NHP)_{73-102}$ suppresses extracellularly regulated kinase (ERK)-1, 2 phosphorylation. A549 cells were transfected with control vector or NHP_{73-102}. Cell lysates 24 hours after transfection were immunoblotted for phospho-ERK-1, 2. Blots were stripped and reprobed for ERK-1, 2. The experiment was repeated 3 times, and a representative experiment is shown.

Although this report describes some specific effects of increased intracellular NHP_{73-102} expression, the precise molecular mechanism underlying the actions of this peptide are unknown. The physiologic significance of NHP_{73-102} has not been proven, but the fact that it has a stretch of 4 amino acids in common with the N-terminus of ANP and a stretch of 8 amino acids in common with the N-terminus of urodilatin (UD), and that both of these regions are highly conserved throughout many species,[46] renders the NHP_{73-102} peptide a potential candidate for use as a therapeutic agent in airway disease. Both ANP and UD have bronchodilatory activity in humans; however, UD seems to have a greater bronchodilatory effect than ANP.[46] Whether, the C-terminal 8 amino acids of NHP_{73-102}, which overlap with UD and ANP, potentiate modulation of the ANP system in vivo remains to be investigated. Alternatively, the critical amino acids within NHP_{73-102} might reside in the region that makes the kaliuretic peptide.

In summary, the results of this report show that NHP_{73-102} possesses both bronchodilatory and anti-inflammatory effects, respectively, by virtue of its ability to produce NO and to decrease activation of NFkB and ERK-1, 2 in human airway epithelial cells.

REFERENCES

1. Vesely DL. Atrial natriuretic peptide prohormone gene expression: hormones and diseases that upregulate its expression. IUBMB Life 2002;53:153-9.
2. Misono KS. Natriuretic peptide receptor: structure and signaling. Mol Cell Biochem 2002;230:49-60.
3. Abbey SE, Potter LR. Lysophosphatidic acid inhibits C-type natriuretic peptide activation of guanylyl cyclase-B. Endocrinology 2003;144:240-6.
4. Silberbach M, Roberts CT Jr. Natriuretic peptide signalling: molecular and cellular pathways to growth regulation. Cell Signal 2001;13:221-31.
5. Gutkowska J, Cantin M, Genest J, Sirois P. Release of immunoreactive atrial natriuretic factor from the isolated perfused rat lung. FEBS Lett 1987;214:17-20.
6. Hulks G, Jardine AG, Connell JM, Thomson NC. Influence of elevated plasma levels of atrial natriuretic factor on bronchial reactivity in asthma. Am Rev Respir Dis 1991;143:778-82.
7. Di Nardo P, Peruzzi G. Physiology and pathophysiology of atrial natriuretic factor in lungs. Can J Cardiol 1992;8:503-8.
8. Lindberg F, Andersson KE. Vasodilator responses to alpha-human-atrial natriuretic peptide in isolated omental and pulmonary arteries from rabbit and man. Acta Physiol Scand 1988;134:391-7.
9. Tolsa JF, Gao Y, Sander FC, Souici AC, Moessinger A, Raj JU. Differential responses of newborn pulmonary arteries and veins to atrial and C-type natriuretic peptides. Am J Physiol Heart Circ Physiol 2002;282:H273-80.
10. Devillier P, Corompt E, Breant D, Caron F, Bessard G. Relaxation and modulation of cyclic AMP production in response to atrial natriuretic peptides in guinea pig tracheal smooth muscle. Eur J Pharmacol 2001;430:325-33.
11. Mikawa K, Kume H, Takagi K. Effects of atrial natriuretic peptide and 8-brom cyclic guanosine monophosphate on human tracheal smooth muscle. Arzneimittelforschung 1998;48:914-8.
12. Robichaud A, Michoud MC, Saunier C, Duvivier C, Peslin R, du Souich P. Effect of atrial natriuretic peptide on bronchial tone in anesthetized rabbits. Peptides 1993;14:1325-30.
13. Ohbayashi H, Suito H, Takagi K. Compared effects of natriuretic peptides on ovalbumin-induced asthmatic model. Eur J Pharmacol 1998;346:55-64.
14. Angus RM, McCallum MJ, Thomson NC. Effect of inhaled atrial natriuretic peptide on methacholine induced bronchoconstriction in asthma. Clin Exp Allergy 1994;24:784-8.
15. Angus RM, McCallum MJ, Hulks G, Thomson NC. Bronchodilator, cardiovascular, and cyclic guanylyl monophosphate response to high-dose infused atrial natriuretic peptide in asthma. Am Rev Respir Dis 1993;147:1122-5.
16. Angus RM, Millar EA, Chalmers GW, Thomson NC. Effect of inhaled atrial natriuretic peptide and a neutral endopeptidase inhibitor on histamine-induced bronchoconstriction. Am J Respir Crit Care Med 1995; 151:2003-5.
17. Mizuguchi M, Myo S, Fujimura M. Bronchoprotective effects of atrial natriuretic peptide against propranolol-induced bronchoconstriction after allergic reaction in guinea pigs. Clin Exp Allergy 2000;30:439-44.
18. Mcalpine LG, Hulks G, Thomson NC. Effect of atrial natriuretic peptide given by intravenous infusion on bronchoconstriction induced by ultrasonically nebulized distilled water (fog). Am Rev Respir Dis 1992;146:912-5.
19. Biollaz J, Callahan LT 3rd, Nussberger J, Waeber B, Gomez HJ, Blaine EH, Brunner HR. Pharmacokinetics of synthetic atrial natriuretic peptides in normal men. Clin Pharmacol Ther 1987;41:671-7.
20. Kumar M, Behera AK, Lockey RF, Vesely DL, Mohapatra SS. Atrial natriuretic peptide gene transfer by means of intranasal administration attenuates airway reactivity in a mouse model of allergic sensitization. J Allergy Clin Immunol 2002;110:879-82.
21. Ishii Y, Watanabe T, Watanabe M, Hasegawa S, Uchiyama Y. Effects of atrial natriuretic peptide on type II alveolar epithelial cells of the rat lung. Autoradiographic and morphometric studies. J Anat 1989;166:85-95.
22. Cvek K, Ridderstrale Y, Gerstberger R. Functional receptors for atrial natriuretic peptide in the rat mammary gland during lactation. Endocrinology 1998;139:2615-21.
23. Demaurex N, Arnaudeau S, Opas M. Measurement of intracellular $Ca2^{+}$ concentration. Methods Cell Biol 2002;70:453-74.
24. Misko TP, Schilling RJ, Salvemini D, Moore WM, Currie MG. A fluorometric assay for the measurement of nitrite in biological samples. Anal Biochem 1993;214:11-6.
25. Holschermann H, Noll T, Hempel A, Piper HM. Dual role of cGMP in modulation of macromolecule permeability of aortic endothelial cells. Am J Physiol 1997;272:H91-8.
26. Howarth PH, Redington AE, Springall DR, Martin U, Bloom SR, Polak JM, et al. Epithelially derived endothelin and nitric oxide in asthma. Int Arch Allergy Immunol 1995;107:228-30.
27. Christman JW, Sadikot RT, Blackwell TS. The role of nuclear factor-kappa B in pulmonary diseases. Chest 2000;117:1482-7.
28. Kook H, Itoh H, Choi BS, Sawada N, Doi K, Hwang TJ, et al. Physiological concentration of atrial natriuretic peptide induces endothelial regeneration in vitro. Am J Physiol Heart Circ Physiol 2003;284:H1388-97.
29. Kiemer AK, Bildner N, Weber NC, Vollmar AM. Characterization of heme oxygenase 1 (heat shock protein 32) induction by atrial natriuretic peptide in human endothelial cells. Endocrinology 2003;144:802-12.
30. Sweeney M, McDaniel SS, Platoshyn O, Zhang S, Yu Y, Lapp BR, et al. Role of capacitative $Ca2^{+}$ entry in bronchial contraction and remodeling. J Appl Physiol 2002;92:1594-602.
31. Liu L, Hakansson L, Ridefelt P, Garcia RC, Venge P. Priming of eosinophil migration across lung epithelial cell monolayers and upregulation of CD11b/CD18 are elicited by extracellular $Ca2^{+}$. Am J Respir Cell Mol Biol 2003;28:713-21.
32. Toda M, Tulic MK, Levitt RC, Hamid Q. A calcium-activated chloride channel (HCLCA1) is strongly related to IL-9 expression and mucus production in bronchial epithelium of patients with asthma. J Allergy Clin Immunol 2002;109:246-50.
33. Kiemer AK, Vollmar AM. Elevation of intracellular calcium levels contributes to the inhibition of nitric oxide production by atrial natriuretic peptide. Immun Cell Biol 2001;79:11-7.
34. Kiemer AK, Vollmar AM. Autocrine regulation of inducible nitricoxide synthase in macrophages by atrial natriuretic peptide. J Biol Chem 1998;273:13444-51.
35. Pawliczak R, Cowan MJ, Huang X, Nanavaty UB, Alsaaty S, Logun C, et al. p11 expression in human bronchial epithelial cells is increased by nitric oxide in a cGMP-dependent pathway involving protein kinase G activation. J Biol Chem 2001;276:44613-21.
36. de Boer J, Meurs H, Flendrig L, Koopal M, Zaagsma J. Role of nitric oxide and superoxide in allergen-induced airway hyperreactivity after the late asthmatic reaction in guinea-pigs. Br J Pharmacol 2001;138:1235-42.
37. Silkoff PE, Robbins RA, Gaston B, Lundberg JO, Townley RG. Endogenous nitric oxide in allergic airway disease. J Allergy Clin Immunol 2000;105:438-48.

38. Folkerts G, Nijkamp FP. Airway epithelium: more than just a barrier! Trends Pharmacol Sci 1998;19:334-41.
39. Hamad AM, Johnson SR, Knox AJ. Antiproliferative effects of NO and ANP in cultured human airway smooth muscle. Am J Physiol 1999;277:L910-8.
40. Calderone A. The therapeutic effect of natriuretic peptides in heart failure; differential regulation of endothelial and inducible nitric oxide synthases. Heart Fail Rev 2003;8:55-70.
41. Kiemer AK, Weber NC, Vollmar AM. Induction of IkappaB: atrial natriuretic peptide as a regulator of the NF-kappaB pathway. Biochem Biophys Res Commun 2002;295:1068-76.
42. Carini R, De Cesaris MG, Splendore R, Domenicotti C, Nitti MP, Pronzato MA, et al. Mechanisms of hepatocyte protection against hypoxic injury by atrial natriuretic peptide. Hepatology 2003;37:277-85.
43. Kiemer AK, Vollmar AM. The atrial natriuretic peptide regulates the production of inflammatory mediators in macrophages. Ann Rheum Dis 2001;60:iii68-70.
44. Kiemer AK, Vollmar AM, Bilzer M, Gerwig T, Gerbes AL. Atrial natriuretic peptide reduces expression of TNF-alpha mRNA during reperfusion of the rat liver upon decreased activation of NF-kappaB and AP-1. J Hepatol 2000;33:236-46.
45. Kiemer AK, Weber NC, Furst R, Bildner N, Kulhanek-Heinze S, Vollmar AM. Inhibition of p38 MAPK activation via induction of MKP-1: atrial natriuretic peptide reduces TNF-alpha-induced actin polymerization and endothelial permeability. Circ Res 2002;90:874-81.
46. Fluge T, Fabel H, Wagner TO, Schneider B, Forssmann WG. Urodilatin (ularitide, INN): a potent bronchodilator in asthmatic subjects. Eur J Clin Invest 1995;25:728-36.