

Brain Natriuretic Peptide Protects against Hyperresponsiveness of Human Asthmatic Airway Smooth Muscle via an Epithelial Cell-Dependent Mechanism

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Abstract

Brain natriuretic peptide (BNP) relaxes airways by activating natriuretic peptide receptor-A and elevating cyclic guanosine monophosphate. BNP is more effective in passively sensitized human bronchi compared with control airways. The molecular and cellular patterns involved in this signaling are unknown. The aim of this study was to investigate the influence of BNP on airway smooth muscle (ASM) cells obtained from donors with asthma and healthy donors and to identify the mechanisms involved in BNP-mediated relaxation. The contractile response of ASM cells was microscopically assessed *in vitro* in the presence of 1 μ M BNP or with supernatant from human bronchial epithelial (BEAS-2B) cells pretreated with 1 μ M BNP. We investigated the role of muscarinic M₂ receptors and inducible nitric oxide synthase (iNOS), quantified the release of acetylcholine and nitric oxide (NO), and assessed the gene/protein expression of iNOS and myosin phosphatase target subunit 1

(MYPT1). Supernatant from BEAS-2B cells treated with BNP reduced the hyperreactivity of asthmatic ASM cells by shifting the potency of histamine by 1.19-fold but had no effect in healthy ASM cells. BNP was not effective directly on ASM cells. Blocking muscarinic M₂-receptors and iNOS abolished the protective role of supernatant from BEAS-2B treated with BNP. BNP stimulated the release of acetylcholine ($210.7 \pm 11.1\%$) from BEAS-2B cells that in turn increased MYPT1 and iNOS gene/protein expression and enhanced NO levels in asthmatic ASM supernatant ($35.0 \pm 13.0\%$). This study provides evidence that BNP protects against bronchial hyperresponsiveness via an interaction between respiratory epithelium and ASM in subjects with asthma.

Keywords: brain natriuretic peptide; bronchial hyperresponsiveness; airway smooth muscle; airway epithelium; nitric oxide

The natriuretic peptide (NP) hormones, a family of cardiac- and vascular-derived peptides, consist of different small proteins characterized by a 17-amino-acid loop and include atrial NP (ANP), brain NP (BNP), and C-type NP (CNP) (1). NPs represent the natural agonist for at the least three homologous single-transmembrane, glycosylated NP receptors (NPRs). NPR-A and NPR-B transduce the NP signal via activation of a large cytoplasmic domain with guanylyl cyclase activity and

consequent production of cyclic guanosine monophosphate (cGMP). NPR-C inhibits adenylyl cyclase and activates phospholipase C. NPR-C is not linked with guanylyl cyclase but serves as a "clearance" receptor to remove NP hormones from the circulation (2, 3).

NPR-A represents the biological receptor for ANP and BNP, and it is expressed on a variety of animal and human respiratory cells, such as type II alveolar cells, airway epithelial cells, and airway

smooth muscle (ASM) cells (4–7). Recent studies have demonstrated that the activation of NPR-A plays a role in relaxing human ASM cells. Thus, the administration of ANP and human recombinant BNP (nesiritide) caused bronchorelaxation in patients with asthma, and the novel NPR-A agonist PL-3994 induced concentration-dependent cGMP production and relaxation of precontracted human precision-cut lung slices. Moreover, we have previously demonstrated that the

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broncho-relaxant activity of BNP was more effective in passively sensitized human isolated bronchi compared with nonsensitized airway tissues (7–11).

Bronchial hyperresponsiveness (BHR) is a central phenotypic feature of patients with bronchial asthma. BHR can be assessed by direct contractile stimuli, such as acetylcholine, histamine, prostaglandins, or leukotrienes, and by indirect stimuli, such as adenosine, tachykinins, exercise, or hyper/hypotonic aerosols. For example, the ASM contraction mediated by adenosine may be partially inhibited by histamine H_1 -receptor antagonists or by cysteinyl leukotriene Cys- LT_1 -receptor antagonists in human isolated bronchi (12). BHR has been suggested to be secondary to the release of inflammatory mediators (13), although a number of novel treatments focused on the modulation of inflammation to influence BHR in asthma have failed clinically, questioning the central role played by airways inflammation in this process (14). This has led to the suggestion that other features that affect lung physiology may be important in the pathogenesis of BHR, such as airway remodeling and changes in the responsive characteristics of ASM (15). However, the intermediary cells between the indirect stimuli and the effector cells, primarily represented by ASM, are also likely structural inflammatory cells in the airway that can release contractile agonists (13). The ability of BNP to relax ASM contraction induced by histamine in human airways suggests that BNP might play a role in modulating BHR and may also indirectly influence the bronchial responsiveness caused by agents that induce the release of histamine (7, 8).

Although previous studies have proved that the broncho-relaxant activity of NP hormones was correlated with the activation of NPR-A, which enhances the concentration of the second messenger cGMP (2, 3), only a few studies have investigated the downstream molecular pathway activated by BNP and the specific cellular pattern involved in this signaling transduction (7). Thus, our previous findings suggest that the activation of NPR-A, expressed on human bronchial epithelium and activated by BNP, was associated with relaxation of isolated bronchi through the stimulation of muscarinic M_2 receptors and a subsequent release of nitric oxide (NO) by underlying

nonepithelial bronchial tissues, including ASM cells. However, the difficulty in interpreting data derived from experiments that used whole isolated sensitized bronchi rather than tissue from subjects with asthma prevented the identification of the specific cellular pathways involved in the BNP response in ASM cells in subjects with asthma in comparison with healthy subjects (7).

Therefore, the aim of this study was to investigate the influence of BNP on ASM cells obtained by bronchial biopsy from asthmatic and healthy donors and to identify the potential downstream mechanisms involved in the BNP-mediated relaxation of ASM cells.

Materials and Methods

ASM and Epithelial Cell Culture

Immortalized normal human bronchial epithelial cells (BEAS-2B) were cultured with LHC-9 and RPMI 1640 medium. Human ASM cells (from healthy subjects without asthma and from patients with asthma) were cultured in supplemented DMEM-growth medium. Cells were maintained at 37°C with an atmosphere of 5% CO_2 and 95% air.

Details for the ASM and epithelial cell culture are reported in the online supplement.

Study Design

The supernatant of BEAS-2B cells treated with 1 μ M BNP for 1 hour was collected. In some experiments, BEAS-2B cells were passively sensitized to mimic *in vitro* characteristics of asthmatic airway hyperresponsiveness *in vivo*.

Human ASM cells were treated as follows. Cells in group A were incubated for 1 hour with diluted (1:10) supernatant from BEAS-2B cells (passively sensitized and nonsensitized) treated with BNP; cells in group B were incubated for 1 hour with 1 μ M BNP; cells in group C were incubated in 0.1 μ M methoctramine (a selective inhibitor of muscarinic M_2 receptor subtypes at nanomolar concentrations) for 30 minutes, followed by incubation for 1 hour with diluted (1:10) supernatant from BEAS-2B cells treated with BNP; and cells in group D were incubated in 0.1 mM aminoguanidine, a selective inhibitor of inducible NO synthase (iNOS), at micromolar concentrations for 30 minutes,

followed by incubation for 1 hour with diluted (1:10) supernatant from BEAS-2B cells treated with BNP (16–18). In control cultures, medium was freshly replaced. After treatments, semilogarithmic concentration response curves (CRCs) with histamine were performed.

In other experiments, the relaxant effect of supernatant from BEAS-2B cells treated with BNP, of salbutamol, and of low concentrations of acetylcholine (0.1 pM to 10 nM) on the contractile response of ASM cells obtained from subjects with asthma was investigated. Further details can be found in the online supplement.

Assessment of ASM Contractility

The contractile response of ASM cells was expressed as a percentage reduction in cell length compared with the initial resting length. Images were taken using a microscope equipped with a charge-coupled device camera. Further details are available in the online supplement.

Determination of Acetylcholine and NO Release

After treatments, supernatant from BEAS-2B and human ASM cell cultures were collected for the quantification of acetylcholine and NO as reported in the online supplement.

MTT Assay

MTT assays confirmed that BNP at 1 μ M was not cytotoxic in BEAS-2B or human ASM cultures. Further details are reported in the online supplement.

Real-Time PCR and Protein Detection

Gene and protein expression for iNOS, myosin phosphatase target subunit 1 (MYPT1), and phosphorylated MYPT1 at Thr696 (pMYPT1^{Thr696}) were assessed by quantitative real-time PCR and Western blot as described in the online supplement.

Statistical Analysis

Curve-fitting to a sigmoidal model was used to calculate the effect (E), the maximal response (E_{max}), the dose inducing a 50% maximal effect (EC_{50}) of the semilogarithmic CRC to histamine, and the negative logarithm of EC_{50} (pEC_{50}) (19, 20). Results were expressed as mean $\pm$ SEM, and statistical significance was assessed by Student's *t* test for $P < 0.05$.

Data and image analyses were performed using GraphPad Prism and ImageJ software (21, 22).

Drugs

Details about the aminoguanidine, BNP, histamine, methoctramine, papaverine, and salbutamol used in this study are reported in the online supplement.

Results

Contractile Response of ASM Cells to Histamine and the Influence of BNP

Histamine (1 nM to 1 mM) induced a sigmoid CRC in nonasthmatic and asthmatic ASM cells. $E_{\max}$ induced by histamine was not significantly different between groups (nonasthmatic cells, $28.07 \pm 3.38 \mu\text{m}$; asthmatic cells, $31.76 \pm 4.98 \mu\text{m}$ [$n = 8$]; $P > 0.05$), whereas its potency was significantly higher in the asthmatic group when compared with nonasthmatic ASM cells (pEC_{50} , 6.36 ± 0.13 and 5.98 ± 0.09 , respectively [$n = 8$]; $P < 0.05$) (Figure 1). Supernatant from BEAS-2B cells used as a negative control failed to induce contraction at any time point and did not modify the contractile response of ASM cells induced by histamine; similarly, the supernatant from BEAS-2B cells treated with BNP did not significantly ($n = 8$; $P > 0.05$) modify the contractility of resting ASM cells ($n = 8$; $P > 0.05$; data not shown).

The supernatant from BEAS-2B cells treated with BNP was weakly effective in relaxing the ASM contraction induced by histamine in cells obtained from subjects with asthma ($E_{\max}$: $50.07 \pm 7.02\%$ at BNP 1 μM ; pEC_{50} : 6.71 ± 0.22), which was similar to the effect of salbutamol ($E_{\max}$: $62.57 \pm 5.28\%$; pD_2 : 6.77 ± 0.17) (Figure 1), but it was ineffective in relaxing ASM cells obtained from healthy donors ($n = 8$; $P > 0.05$; data not shown). In the following experiments we used BNP at 1 μM because this was the concentration inducing the relaxant $E_{\max}$.

Incubation for 1 hour with diluted (1:10) supernatant from BEAS-2B cells treated with 1 μM BNP did not modify ($n = 8$; $P > 0.05$) the pEC_{50} of histamine in nonasthmatic ASM cells but significantly ($n = 8$; $P < 0.001$) shifted the CRC induced by histamine in asthmatic ASM cells (pEC_{50} : nonsensitized BEAS-2B cells, 5.17 ± 0.17 ; passively sensitized BEAS-2B cells,

4.83 ± 0.10) to the right when compared with control cells (Figures 1 and 2), although there was no time-dependent effect as assessed by treating cells for 1 to 3 hours ($n = 8$; $P > 0.05$). The passive sensitization of BEAS-2B cells did not alter the protective effect of BNP on the contractile response of asthmatic ASM cells induced by histamine compared with the effect mediated by nonsensitized BEAS-2B cells ($n = 8$; $P > 0.05$) (Figure 1).

The study of ASM cell contractility has been focused on the influence of 1 μM BNP and BEAS-2B cells on the contractile response induced by histamine in asthmatic ASM cells. BNP (1 μM) did not significantly ($n = 8$; $P > 0.05$) alter the CRC to histamine. However, antagonizing muscarinic M_2 receptors with 0.1 μM methoctramine and inhibiting iNOS with 0.1 mM aminoguanidine abolished the protective effect of incubation with the supernatant from BEAS-2B cells treated with 1 μM BNP on the contractile response to histamine in asthmatic ASM cells (Figure 1).

Influence of BNP on the Release of Acetylcholine and NO

The treatment of BEAS-2B cells with 1 μM BNP stimulated the release of acetylcholine ($210.66 \pm 11.05\%$ [$n = 3$]; $P < 0.001$) compared with controls. This effect was not seen with ASM cells ($5.21 \pm 7.85\%$ [$n = 3$]; $P > 0.05$). In addition, 1 μM BNP did not significantly modulate the release of NO from BEAS-2B cells ($n = 3$; $P > 0.05$). We therefore investigated the potential relaxant effect of acetylcholine and observed that acetylcholine at very low concentrations (at the picomolar and nanomolar range) was able to weakly relax ASM cells from donors with asthma submaximally precontracted with histamine ($E_{\max}$: $-21.52 \pm 2.73\%$) (Figure 1).

Because the incubation with supernatant from BEAS-2B cells treated with 1 μM BNP protected asthmatic ASM cells, but not nonasthmatic cells, against the contractile response induced by histamine, we investigated the release of NO from asthmatic ASM cells after different pharmacological treatments.

The direct treatment of asthmatic ASM cells with 1 μM BNP did not significantly modulate NO concentrations ($-4.0 \pm 3.0\%$ [$n = 3$]; $P > 0.05$), whereas the incubation with supernatant from BEAS-2B cells treated with 1 μM BNP significantly enhanced NO concentrations in the

supernatant (nonsensitized BEAS-2B cells $35.0 \pm 13.0\%$; passively sensitized BEAS-2B cells [$n = 3$], $49.0 \pm 8.5\%$; $P < 0.01$) compared with control cells. Furthermore, the passive sensitization of BEAS-2B cells did not modify the release of NO by asthmatic ASM cells compared with the effect mediated by nonsensitized BEAS-2B cells ($n = 3$; $P > 0.05$) (Figure 3). The administration of 0.1 μM methoctramine and 0.1 mM aminoguanidine to block muscarinic M_2 receptors and to inhibit iNOS, respectively, abolished the release of NO mediated by the incubation with supernatant from BEAS-2B cells treated with BNP (1 μM) compared with control cells. The same treatments did not significantly modify the release of NO in ASM from healthy donors compared with control cells ($n = 3$; $P > 0.05$) (Figure 3).

The Influence of BNP on iNOS Activity in Asthmatic ASM Cells

The iNOS mRNA and protein expression of asthmatic ASM cells followed the same course as the release of NO under the same pharmacological conditions. In fact, the expression of iNOS mRNA was not modulated by the direct incubation of asthmatic ASM cells with 1 μM BNP ($-18.50 \pm 7.50\%$ [$n = 3$]; $P > 0.05$), whereas the incubation with supernatant from BEAS-2B cells treated with 1 μM BNP significantly enhanced the iNOS transcript level ($62.96 \pm 5.00\%$ [$n = 3$]; $P < 0.001$) compared with control cells. Antagonizing muscarinic M_2 receptors and inhibiting iNOS with 0.1 μM methoctramine and 0.1 mM aminoguanidine, respectively, completely inhibited the increase of iNOS transcript levels mediated by the incubation with supernatant from BEAS-2B cells treated with 1 μM BNP compared with control cells (Figure 3).

The iNOS protein expression significantly increased after treating ASM cells with supernatant from BEAS-2B cells treated with 1 μM BNP ($50.59 \pm 5.02\%$ [$n = 3$]; $P < 0.05$), and blocking M_2 receptors with 0.1 μM methoctramine prevented this phenomenon ($P > 0.05$; $n = 3$) compared with control cells (Figure 4).

Supernatant from BEAS-2B Cells Treated with BNP Increases MYPT1 Gene Transcription and Protein Expression in Asthmatic ASM Cells

We analyzed gene transcription and protein expression of MYPT1, a regulatory subunit

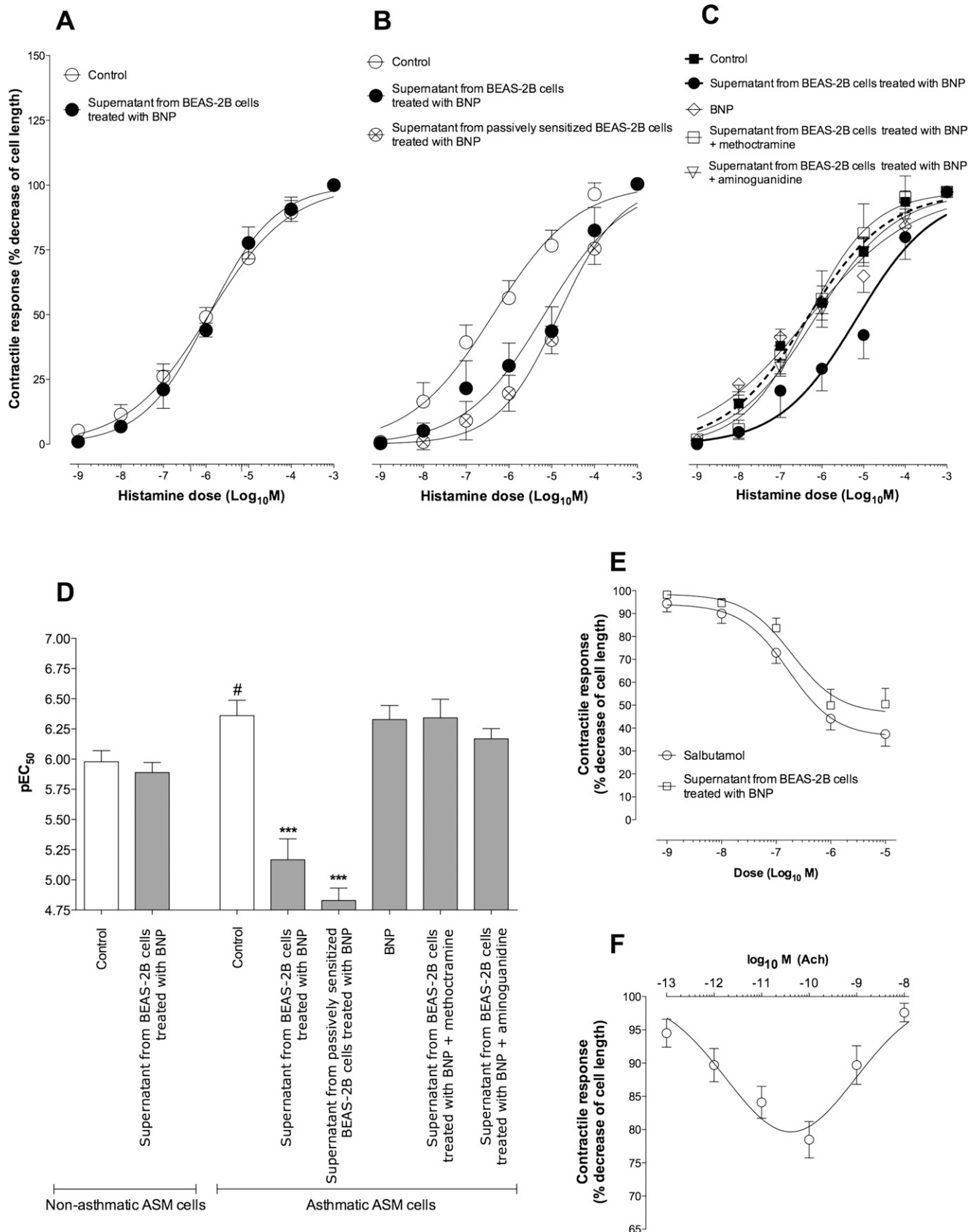


Figure 1. (See figure legend on following page)

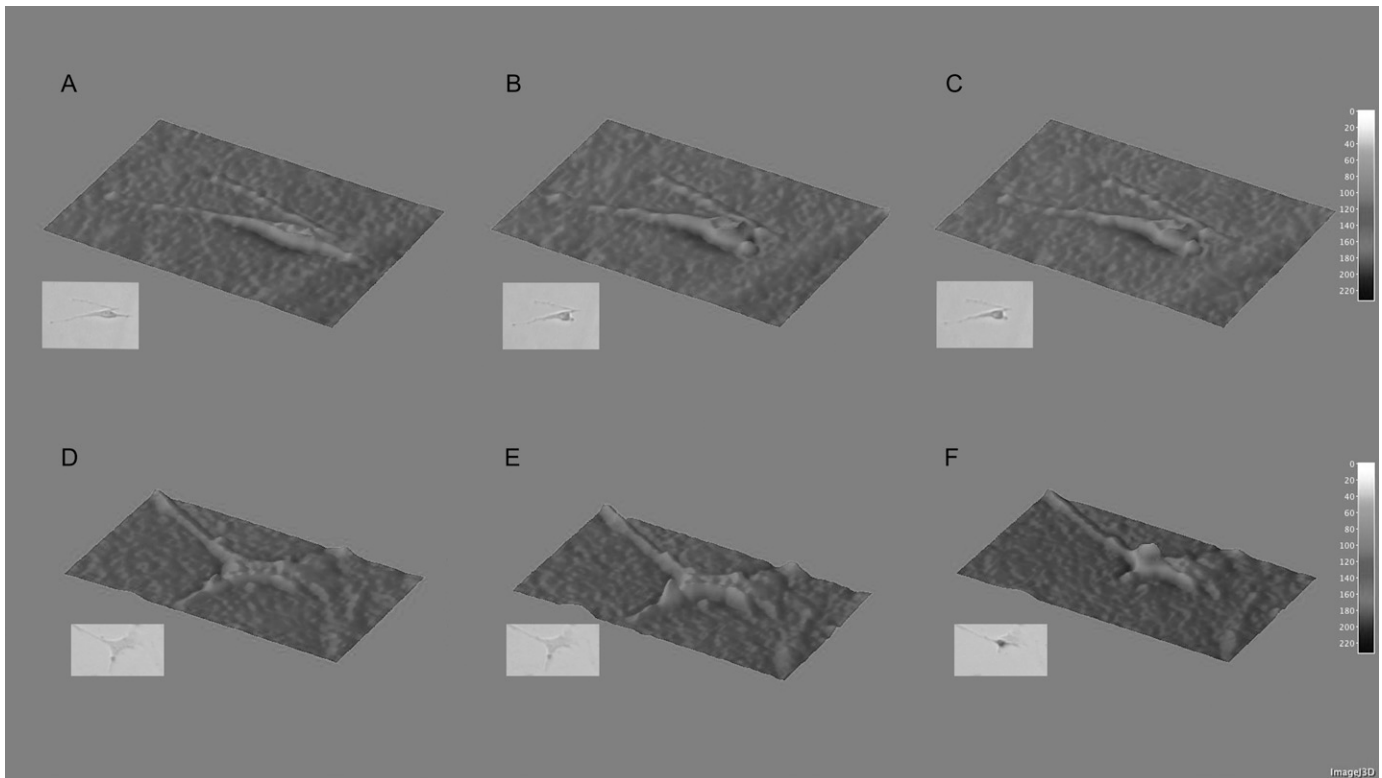


Figure 2. Interactive three-dimensional surface plot representation of contractile response of asthmatic ASM cells to histamine (*B*, *C*, *E*, *F*) and control cells (*A* and *D*). Histamine (10 μ M) (*B*) induced a submaximal contractile response ($\approx$ 80% maximal response respect to 1 mM histamine [*C*]), whereas the treatment with supernatant from BEAS-2B cells prestimulated with 1 μ M BNP (*E*) prevented the contractile response of asthmatic ASM cells ($\approx$ 40% maximal response respect to 1 mM histamine [*F*]). The interactive three-dimensional surface translates the luminance of two-dimensional images (*small pictures*) as height for the plot using the nearest neighbor sampling.

of myosin light chain phosphatase, with an inactive phosphorylated form represented by pMYPT1^{Thr696} that plays a key role in the regulation of smooth muscle tone (23). Unstimulated asthmatic ASM cells displayed a low MYPT1 mRNA transcript level that was not modulated by direct incubation with 1 μ M BNP ($n = 3$; $P > 0.05$). Instead, the incubation of asthmatic ASM cells with supernatant from BEAS-2B cells treated with 1 μ M BNP enhanced

the mRNA level by 10 ± 1 -fold ($n = 3$; $P < 0.01$) compared with unstimulated asthmatic cells and other experimental conditions (Figure 3). MYPT1 transcript levels in asthmatic ASM cells treated with 0.1 μ M methoctramine and 0.1 mM aminoguanidine were similar to that of control cells.

The supernatant from BEAS-2B cells treated with 1 μ M BNP significantly reduced the pMYPT1^{Thr696} ($-64.94 \pm$

2.88% [$n = 3$]; $P < 0.05$) compared with control cells, and antagonizing M_2 receptors with 0.1 μ M methoctramine significantly prevented this phenomenon ($P < 0.01$; $n = 3$). The supernatant from BEAS-2B cells treated with BNP (1 μ M) also significantly enhanced the protein expression of the unphosphorylated active form of MYPT1 by 4.19 ± 0.19 -fold ($n = 3$; $P < 0.01$) compared with control cells (Figure 4).

Figure 1. (Continued). Concentration–response curves of histamine in nonasthmatic (*A*) and asthmatic (*B*) airway smooth muscle (ASM) cells and influence of the pretreatment with diluted (1:10) supernatant from human epithelial cells (BEAS-2B) stimulated 1 hour with 1 μ M brain natriuretic peptide (BNP). In some experimental groups, the supernatant was collected from passively sensitized BEAS-2B cells. (*C*) Concentration–contraction curves of histamine in asthmatic ASM cells and influence of 1 μ M BNP and of diluted (1:10) supernatant from BEAS-2B cells prestimulated with 1 μ M BNP. In some experimental groups muscarinic M_2 receptor subtype was selectively blocked with 0.1 μ M methoctramine, and inducible nitric oxide synthase (iNOS) was inhibited with 0.1 mM aminoguanidine. The *bar graph* shows histamine potency (pEC_{50}) (*D*) on the contractile response of nonasthmatic and asthmatic ASM cells. ASM cells were pretreated before the administration of histamine with diluted (1:10) supernatant from BEAS-2B cells (passively sensitized and nonsensitized) stimulated with BNP at 1 μ M concentration; 1 μ M BNP; 0.1 μ M methoctramine (a selective inhibitor of muscarinic M_2 receptor subtype) followed by incubation with diluted (1:10) supernatant from BEAS-2B cells stimulated with BNP at 1 μ M concentration; and 0.1 mM aminoguanidine (a selective inhibitor of iNOS) followed by incubation with diluted (1:10) supernatant from BEAS-2B cells stimulated with 1 μ M BNP. Relaxant response on the submaximal contraction induced by histamine (EC_{70}) in asthmatic ASM cells by salbutamol and supernatant from BEAS-B cells treated with 1 nM to 10 μ M BNP (*E*) and by acetylcholine at low concentrations (*F*). Each *bar* represents the mean $\pm$ SEM of image analysis performed on eight cells. $^{\#}P < 0.05$ versus nonasthmatic control group; $^{***}P < 0.001$ versus asthmatic control group.

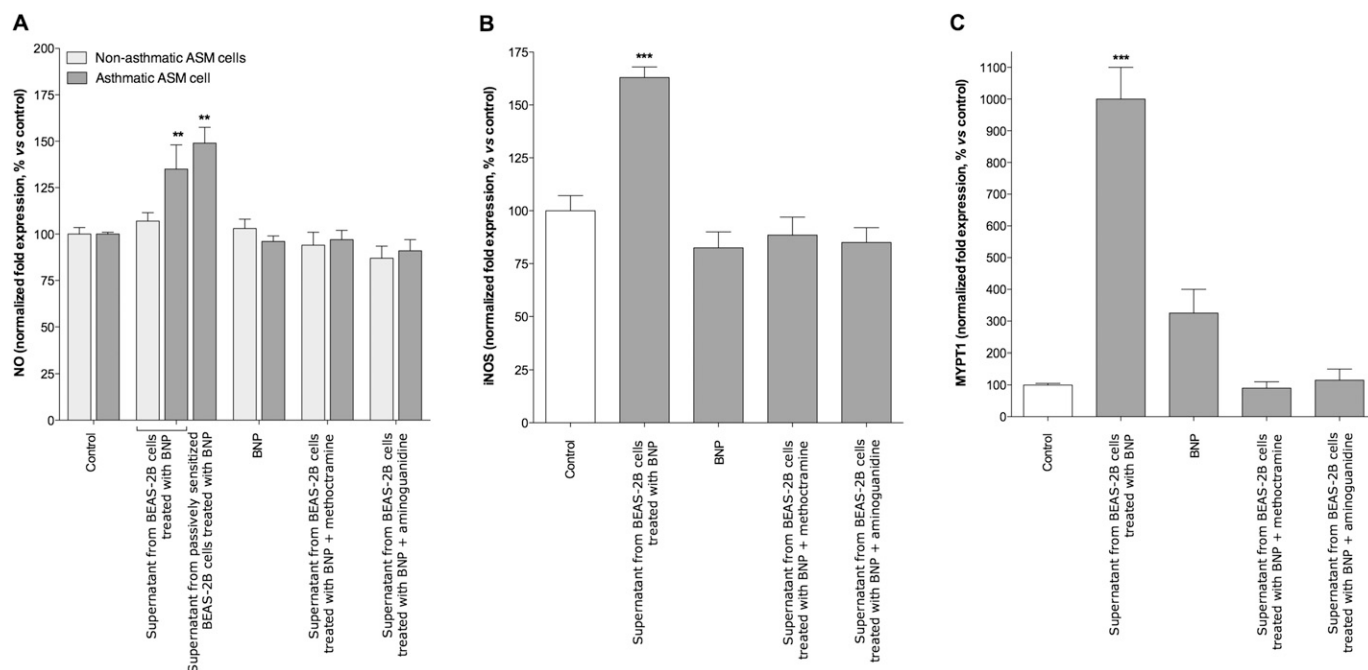


Figure 3. Nitric oxide (NO) levels (A), iNOS gene expression (B), and myosin phosphatase target subunit 1 (MYPT1) gene expression (C) by real-time PCR in ASM cells from donors with asthma and nonasthmatic donors (A) and from donors with asthma (B and C) treated with 1 μ M BNP and with diluted (1:10) supernatant from BEAS-2B cells prestimulated with 1 μ M BNP. In some experimental groups, 0.1 μ M methoctramine concentration and 0.1 mM aminoguanidine were used for inhibiting muscarinic M_2 receptor subtype and iNOS, respectively, and the supernatant was collected from passively sensitized BEAS-2B cells. Values (shown as percentage of control = 100%) are the mean $\pm$ SEM of experiments performed in triplicate. ** P < 0.01 and *** P < 0.001 versus control groups. iNO, inducible NO.

Discussion

In the present study, we have demonstrated that ASM cells from patients with asthma are more sensitive to the contractile effects of histamine compared with ASM cells from nonasthmatic donors. We have also documented that the contractile hyperresponsiveness of asthmatic ASM cells to histamine is completely reversed to a level of response described in healthy ASM cells by BNP involving post-transductional pathways in bronchial epithelial cells. Our results suggest that BNP is unable to directly modulate the contractile response of asthmatic ASM cells, whereas their incubation with supernatant from BEAS-2B cells pretreated with BNP is effective in reducing the contractile response to histamine.

These results are consistent with those previously presented from our laboratories (7, 8, 24) and are in line with the hypothesis that epithelial cells might mediate the BNP-induced relaxant activity in human isolated bronchi through an autocrine loop involving the releasing of acetylcholine and NO. Because these

previous studies were performed using human isolated bronchi from nonasthmatic donors, we could not discern the pathway activated by NPR-A and the link between epithelial cells and ASM cells present in subjects with asthma. However, we were able to demonstrate, with immunohistochemical techniques, that NPR-A receptors were present in bronchial epithelial cells and in inflammatory cells of the lamina propria, but immunodetection of this receptor was faint in the underlying ASM cells (7).

Results from the present study show that the protective role of BNP on the contractile response of ASM to histamine is not mediated by a direct action of BNP on ASM cells obtained from subjects with asthma, but is the result of an interaction between BNP and respiratory epithelial cells that release an endogenous mediator, which we have identified as acetylcholine, released at picomolar concentrations by BEAS-2B cells after treatment with BNP. In effect, as we have previously demonstrated in experiments performed in isolated bronchi (7), acetylcholine weakly relaxes ASM cells from subjects with asthma when

administered at very low concentrations. The protective role of BNP was prevented by antagonizing muscarinic M_2 receptors with adequate concentrations of methoctramine on asthmatic ASM cells. We also demonstrated that the activation of this receptor, through acetylcholine released into the supernatant of BEAS-2B cells treated with BNP, enhanced the concentrations of NO in ASM cells obtained from patients with asthma, but not in ASM cells from healthy subjects, in agreement with previous studies that suggest that patients with atopic asthma show increased levels of exhaled NO compared with healthy control subjects (25, 26). On the other hand, BNP had no direct effect on the NO-releasing capability of asthmatic ASM cells. Furthermore, the inhibition of iNOS by treating asthmatic ASM cells with aminoguanidine prevented the protective effect of supernatant obtained from BEAS-2B cells previously treated with BNP against the contractile hyperreactivity to histamine. The 0.1 mM aminoguanidine used in this study to inhibit iNOS would only cause negligible inhibition of eNOS (16), and our findings

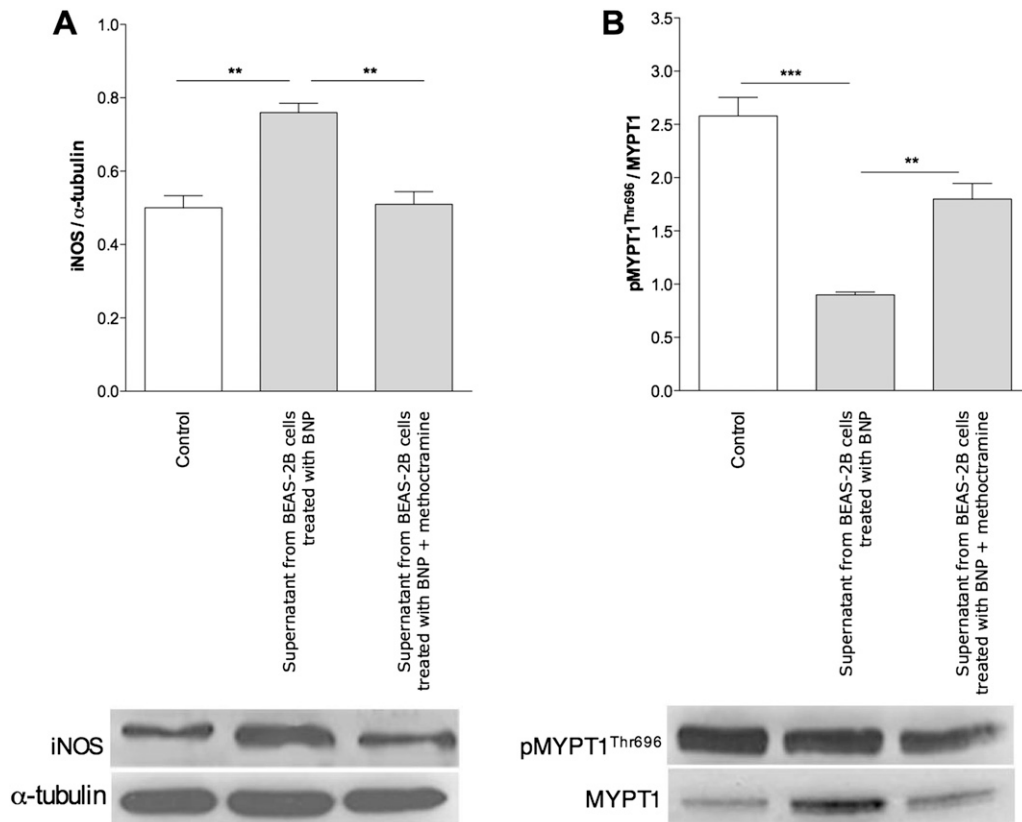


Figure 4. Representative Western blot and densitometric analysis of iNOS (A) and MYPT1 (B) (values expressed as pMYPT1^{Thr696}/MYPT1 ratio) protein expression in human ASM cells from donors with asthma and influence of the pretreatment with diluted (1:10) supernatant from human epithelial cells (BEAS-2B) stimulated for 1 hour with 1 μ M BNP. The role of muscarinic M₂ receptor subtype was assessed by selectively blocking receptors with 0.1 μ M methoctramine. Membranes were reprobed with anti- α -tubulin to ensure equal loading of samples. Each bar represents the mean $\pm$ SEM of Western blot band densitometry analysis detected in triplicate. ** P < 0.01 and *** P < 0.001 versus selected bars.

also suggest that the supernatant of BEAS-2B cells previously stimulated with BNP enhanced the iNOS mRNA level and the iNOS protein expression of asthmatic ASM cells and that treatment with methoctramine and aminoguanidine prevented the increase of iNOS transcription.

ASM tone is determined by the relative activities of myosin light chain kinase and myosin light chain phosphatase, a trimeric enzyme with a catalytic subunit represented by MYPT1. The NO-mediated relaxation of ASM is controlled by the activation of a soluble pool of guanylate cyclase that results in an increase of intracellular cGMP concentrations and cGMP-dependent protein kinase activity. In turn, cGMP-dependent protein kinase activates a number of targets, including myosin light chain phosphatase of smooth muscle, and it has been demonstrated that the level of expression for MYPT1 fine tunes this process (27, 28). The findings of

our study led us to hypothesize that BNP induces an epithelium-mediated relaxant effect through the modulation of the NO/cGMP signaling in ASM cells. Our experiments proved that the supernatant from BEAS-2B cells treated with BNP increases the protein expression of the unphosphorylated and active form of MYPT1 whereas preventing the protein expression of the phosphorylated and inactive pMYPT1^{Thr696} form, at least in ASM cells collected from patients with asthma.

This study demonstrates that BNP exhibits a functional antagonistic effect on the hyperresponsiveness of asthmatic ASM cells stimulated with histamine, although we have previously provided findings for its protective role on the contractile response mediated by the cholinergic pathway (7).

Considering the indirect mechanisms leading to the BHR in subjects with asthma and the important role of histamine as an inflammatory mediator inducing

contractile responses of ASM cells in patients with asthma, our data offer evidence for the effectiveness of BNP as a protective agent against histamine-induced contraction of ASM (13, 15). We have also demonstrated that the relaxant effect of the pathway modulated by BNP was similar to the relaxation induced by salbutamol in ASM cells from asthmatic donors.

Our results are consistent with previous studies performed in animals that suggested an indirect relaxant activity of NPs on ASM cells, but our results demonstrated more than one logarithm reduction in the potency of histamine to contract asthmatic ASM cells compared with the weak bronchorelaxation observed with NPs in rats and guinea pigs (29). Furthermore, it has been proposed that the ability of NPRs to influence human ASM cells in culture is based on the ability of these agents to increase cGMP levels (31). However, our findings exclude any correlation between

these receptors expressed on asthmatic ASM cells and the bronchorelaxant activity of BNP, but confirm that the effect of BNP involves the cooperation between bronchial epithelium and ASM cells. Furthermore, our data confirm the findings of Kummer and colleagues (30) and Wessler and Kirkpatrick (31) concerning the release of acetylcholine from airway epithelial cells and the evidence provided by Sterin-Borda and colleagues (33) related to the increased of NO concentrations mediated by the activation of postsynaptic M_2 muscarinic receptors, at the least in rat atria. The paradoxical relaxant activity on asthmatic ASM cells of low concentrations of acetylcholine, released in the supernatant from BEAS-2B cells treated with BNP, confirms our previous studies in human isolated bronchi (7).

There is debate about using inhaled long-acting muscarinic antagonists for asthma treatment, and their use is not discussed in guidelines for the treatment of asthma (33). However, beneficial effects of tiotropium bromide maintenance dosing in patients with asthma have been reported, and adding tiotropium bromide to a high dose of inhaled corticosteroids combined with long-acting β_2 -agonists improves

airway patency in patients with severe persistent asthma (34–36). These findings confirm the potential role of long-acting muscarinic antagonists in the treatment of asthma and highlight the needed of developing more selective muscarinic M_3 versus M_2 receptor antagonists.

Our results, together with the previous evidence discussed above, confirm the presence of an autocrine loop between epithelial and ASM cells in subjects with asthma that can be induced by BNP. The enhancement of transcript levels of iNOS by BNP suggests a possible long-lasting bronchoprotective activity of BNP against contractile stimuli and may represent an important protective mechanism regulating BHR.

The current mainstream treatment of asthma is focused on the use of inhaled corticosteroids and β_2 -adrenergic receptor agonists. However, there remains considerable interest in better understanding the mechanisms contributing to the pathophysiology of asthma, particularly because current therapy is not able to normalize the BHR, an important goal for improving treatment of this common condition in the future. A number of novel approaches to activate NPRs to elicit bronchodilation have

been attempted, including intravenous infusion or nebulization of ANP, inhaled administration of human recombinant BNP (nesiritide), and the novel NPR-A agonists (PL-3994) (9, 11, 37). Although these new compounds might elicit a potent concentration-dependent, and sometimes long-lasting, relaxant activity of human bronchi, their clinical effectiveness has been limited by their ability to relax vascular smooth muscle. A better understanding of the sensitive autocrine loop generated between airway epithelium and underlying ASM may lead to novel approaches to exploiting this important bronchorelaxant pathway in subjects with asthma.

In conclusion, our study provides further evidence for a role of the BNP pathway in controlling BHR and suggests that administration of BNP or analogs may be of value in the treatment of chronic airway diseases characterized by excessive bronchoconstriction. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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Online data supplement

Brain natriuretic peptide protects against hyperreactivity of human asthmatic airway smooth muscle via an epithelial cell dependent mechanism

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Materials and methods

Demographic characteristics of donors of Airway smooth muscle (ASM)

We used ASM cells from two mild asthmatic donors (both males, 22 years old, , non-smokers, not receiving glucocorticosteroid treatment) and one healthy subject (male, 20 years old and non-smoker).

Airways smooth muscle and epithelial cells culture

Normal human bronchial epithelial cells (BEAS-2B), derived from human bronchial epithelium and immortalized using the adenovirus 12-simian virus/40 hybrid virus (a gift from Dr L. Petecchia, Pulmonary Diseases Unit, G. Gaslini Institute, Genoa, Italy) were cultured with a 1:1 mixture of LHC-9 (Gibco) and RPMI 1640 medium (EuroClone; Milan, Italy). Cells were maintained at 37°C in a 5% CO₂ humidified incubator. BEAS-2B cell lines have been reported to be one of the most widely used cell lines for the studies of epithelial cell biology, pathologic processes, drug metabolism and for studies of airway epithelial cell structure and function, including phenotyping and cytokine regulation, regulation of glucocorticosteroid receptors and response to challenges such as tobacco smoke, particles and hyperoxia (1).

Human ASM cells were obtained in accordance with procedures approved by the Guy's and St. Thomas' Hospitals' Research Ethics Committee (study #05/Q0704/72 and study #08/H0804/67) from bronchial biopsies obtained from one non-asthmatic and clinically healthy subjects and two asthmatic

subjects using methods previously described (2). Cell passages 3-6 were used in all experiments. ASM bundles were visualized using a dissecting microscope and dissected free of surrounding tissue using methods previously described (3). ASM cells were cultured in DMEM-growth medium (supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 1 mM sodium pyruvate, 2mM L-glutamine, 1x non-essential amino acid mixture). Cells were maintained at 37 °C and in 5% CO₂.

The presence of ASM in cultured cells was confirmed by fluorescent immunocytochemistry in near-confluent, fetal bovine serum (FBS)-deprived ASM cells (passage 2) stained (>95%) for smooth muscle-specific α-actin, desmin and calponin to exclude contamination of the culture by fibroblasts or epithelial cells (3). For immunofluorescence, sparse ASM cells were fixed in iced methanol for 5 min. and then incubated with specific antibodies and Hoechst for nuclear staining as previously reported (4) (Figure E1).

Study design

BEAS-2B cells were seeded at 1,400 cells/cm² density, maintained in culture and treated, at approximately 80% of confluence, with 1 µM BNP for 1 hour. In control cultures, substitution with fresh medium was performed. After stimulation with BNP the supernatant was collected.

Human ASM cells were seeded at 7,000 cells/cm² density and starvation was performed for 24 hours. After that, human ASM cells were treated as follows: Group A) - incubation for 1 hour with diluted (1:10) supernatant from BEAS-2B cells treated with BNP; Group B) - incubation for 1 hour with 1 µM BNP;

Group C) - 0.1 μ M methoctramine (a selective inhibitor of the muscarinic M₂ receptor subtype at nM concentrations) for 30 min, followed by incubation for 1 hour with diluted (1:10) supernatant from BEAS-2B cells treated with BNP; Group D) - 0.1 mM aminoguanidine (a selective inhibitor of inducible nitric oxide synthase, iNOS, at μ M concentrations) for 30 min, followed by incubation for 1 hour with diluted (1:10) supernatant from BEAS-2B cells treated with BNP (5-7). In control cultures, medium was freshly replaced. The concentrations of antagonists or inhibitors were chosen on the basis of their half maximal inhibition concentration (IC₅₀) and/or binding affinities (pKi) values previously reported in literature (5, 8-11).

After treatments, semi-logarithmic concentration response curves (CRC) with histamine ranging from 1 nM to 1 mM were performed in order to evaluate the contractile response of human ASM cells.

In further experiments BEAS-2B cells were treated with BNP at concentrations ranging from 1 nM to 10 μ M for 1 hour and the supernatant was collected. After that, human ASM cells from both healthy and asthmatic donors were sub-maximally contracted (EC₇₀) with histamine. When the contractile response reached the plateau, relaxant CRC with diluted (1:10) supernatant from BEAS-2B cells treated with BNP was carried out. At the end of the experiment, papaverine (100 μ M) was then added. The time-dependent effect of the supernatant obtained from BEAS-2B cells treated with BNP and the relaxant response of salbutamol on ASM cells was also assessed. In some experiments the effect of supernatant from BEAS-2B cells treated with BNP was assessed on the resting ASM cells contractility.

Passive sensitization

In some experiments, BEAS-2B cells were passively sensitized *in vitro* in order to closely mimic important characteristics of asthmatic airway hyperreactivity *in vivo* (12). This model was used to study the protective effect of BNP against the contractile response induced by histamine in ASM cells from asthmatic donors and the potential role of NO.

When BEAS-2B cells grew to approximately 70%, were incubated overnight at 37°C in a 5% CO₂ humidified incubator with 1:1 mixture of LHC-9 (Gibco) and RPMI 1640 medium (EuroClone; Milan, Italy) in the absence (non-sensitized control BEAS-2B cells) or presence of 2% vol⁻¹ sensitizing serum (passively sensitized BEAS-2B cells). The serum was prepared by centrifugation of whole blood from patients suffering from an exacerbation of atopic asthma (total IgE >250 Uml⁻¹ specific against common aeroallergens) (13-19). Sera were frozen at -80°C in 200 ml aliquots until required.

Assessment of ASM contractility

The contraction of ASM cells maintained adherent to the culture dish was evaluated at 37 °C using digital time-lapse video microscopy. Acute contraction was elicited within 5 minute of adding the agonist and, for each concentration administered, the cell length contraction/relaxation was measured and expressed as a percentage reduction/enhancement in cell length (effect, E) compared to the maximal cell length reduction (E_{max}). All the cells with a well-defined outline present in a given field were measured (20, 21).

The following equation was used for the quantification of the contractile response of ASM cells:

$$\text{ASM cells contractile response (\%)} = \frac{E}{E_{\max}} * 100$$

where:

$E = (\text{resting length}) - (\text{length after treatment administration})$

$E_{\max} = (\text{resting length}) - (\text{length at } E_{\max})$

Negative controls consisted of administration of medium alone. Images were taken using a microscope with a 40X magnification objective (E600 Eclipse, Nikon). A charge-coupled device camera (1.4 megapixel CCD) and commercial software (ACT-1 package) were used to acquire the images (22-24).

Determination of acetylcholine and nitric oxide release

After treatments, supernatant from BEAS-2B and human ASM cell cultures were collected for the quantification of both acetylcholine and NO by colorimetric assay kits (BioCat GmbH, Heidelberg, Germany) in 96-well plates at 570 or 540 nm, respectively, according to manufacturers' instructions (25).

MTT assay

In order to verify any cytotoxic effect of BNP, an MTT (3-[4,5-dimethylthiazol-2-yl]2,5-diphenyl tetrazolium bromide) assay was performed as previously reported (4, 26). Cytotoxic potency evaluated by the “ALLFIT” computer program documented that BNP at 1 μ M was not cytotoxic in both BEAS-2B and human ASM cultures.

Real-time-polymerase chain reaction

After treatments, human ASM cells were homogenized and pooled total RNA extracted by using TRIzol™ reagent (Invitrogen) (27). A qualitative gene expression profile of iNOS was checked by quantitative Real-Time PCR and performed twice for each independently obtained total RNAs samples, using specific primers as previously reported. (28). For human myosin phosphatase target subunit 1 (MYPT1) we used the following primer pairs: Forward 5'-AAC GAG CAG CTG AAA CGC TG- 3', Reverse 5'- TTG GCG TAA TTG ATG TCG GC as previously reported (29).

Protein isolation and western blot analysis

After extraction and quantification of total cell lysates, proteins were separated by gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted to nitrocellulose transfer membranes and incubated with rabbit polyclonal anti-iNOS (1:200, Thermo Scientific, Pierce Biothechnology), anti-

MYPT1(1:750, Cell Signaling Technology, Beverly, MA), anti-phosphorylated MYPT1(pMYPT1^{Thr696}) (1:750, Cell Signaling Technology, Beverly, MA) and anti- α -tubulin antibody (1:2000, Sigma-Aldrich) followed by horseradish peroxidase conjugate goat anti-rabbit or goat anti-mouse IgG (1:5000, Pierce, Rockford, USA). Densitometric blot analysis was performed in three independent experiments and the level of pMYPT1^{Thr696}/MYPT1 was measured (27).

Statistical analysis

Appropriate curve-fitting to a sigmoidal model was used to calculate the effect (E), the maximal response ($E_{\max}$) and the dose inducing 50% maximal effect (EC_{50}) of the semi-logarithmic CRC to histamine. The equation used was $Y=100/(1+10^{((\text{Log}EC_{50}-X)*\text{HillSlope}))}$ (30, 31). $E/E_{\max}$, indicating the contractile response, was expressed as a percentage reduction in cell length elicited by histamine compared to the initial resting length and EC_{50} values were converted to negative logarithmic values (pEC_{50}) for statistical analysis (30). Untreated BEAS-2B and human ASM cell cultures were used as time controls.

The results are presented as normalized fold expression of triplicate experiments and expressed as mean $\pm$ SEM. Statistical significance was assessed by Student's *t*-test and the level of statistical significance was defined as $P<0.05$.

All data and image analyses were performed using computer software GraphPad Prism (GraphPad Software, San Diego California USA) and ImageJ (National Institutes of Health, Bethesda, Maryland, USA) (32, 33).

Drugs

The following compounds were used in this study: aminoguanidine, BNP, histamine, methoctramine, papaverine and salbutamol. All substances were obtained from Sigma-Aldrich (St. Louis, USA) and were dissolved in distilled water. Compounds were stored in small aliquots at -80°C until their use.

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Figure legend

Figure E1: Morphological and phenotypic features of cultured ASM cells from asthmatic donors. Left, phase contrast micrograph of sixth passage ASM; right, smooth muscle α -actin expression by immunofluorescence in ASM cells and Hoechst nuclear staining; scale bar = 50 μ m.

