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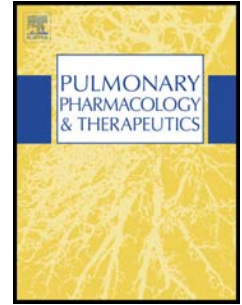
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# Relaxant effect of brain natriuretic peptide in nonsensitized and passively sensitized isolated human bronchi

Maria Gabriella Matera\*, Luigino Calzetta<sup>¤</sup>, Vincenzo Parascandolo+, Giacomo  
Curradi#, Paola Rogliani#, Mario Cazzola#

\*Department of Experimental Medicine, 2<sup>nd</sup> University of Naples, Naples, Italy;  
<sup>¤</sup>The Sackler Institute of Pulmonary Pharmacology, King's College London,  
London, UK; +Department of Thoraco-Pulmonary Surgery, 2<sup>nd</sup> University of  
Naples, Italy; #Department of Internal Medicine, University of Rome 'Tor  
Vergata', Rome, Italy

Correspondence: Prof. Mario Cazzola, Cattedra di Malattie Respiratorie,  
Dipartimento di Medicina Interna, Università di Roma 'Tor Vergata', Via  
Montpellier 1, 00133 Rome, Italy email: [mario.cazzola@uniroma2.it](mailto:mario.cazzola@uniroma2.it)

## Abstract

Brain natriuretic peptide (BNP) relaxes guinea pig tracheal smooth muscle *in vitro* and is effective in preventing ovalbumin-induced bronchoconstriction and microvascular leakage in guinea pigs *in vivo*. Nonetheless, published studies on BNP in human airways *in vitro* are still lacking in the literature. The aim of this study was to investigate the effect of BNP in isolated human bronchi. The relaxant effect of BNP (1nM to 10 $\mu$ M) was assessed in nonsensitized and in passively sensitized human bronchial airways pre-contracted with submaximal concentration (EC<sub>70</sub>) of carbachol or histamine. At the end of the experiment, papaverine (500  $\mu$ M) was then added. BNP induced a weak relaxant activity on carbachol-contracted bronchi in nonsensitized (relaxation: 4.23 $\pm$ 0.51 %) and passively sensitized bronchi (relaxation: 11.31 $\pm$ 2.22 %). On the other hands, BNP induced a relaxant activity on His-contracted bronchi in nonsensitized (relaxation: 42.52 $\pm$ 9.03 %) and in passively sensitized (relaxation: 60.57 $\pm$ 9.58 %). All these findings are a clear documentation of the modest relaxant role of BNP in asthma and, likely, COPD.

**Keywords:** brain natriuretic peptide, carbachol, histamine, human airways, isolated bronchi, passive sensitization

## Introduction

Atrial natriuretic peptide (ANP), B-type natriuretic peptide (BNP) and C-type natriuretic peptide (CNP) are members of the natriuretic peptide family of bioactive peptides best known for their role in the regulation of blood pressure and cardiovascular homeostasis [1, 2]. The cellular responsiveness of natriuretic peptides is manifested through the specific cell surface receptors in different target tissues [1]. Cloning and expression of cDNAs led to the identification and characterization of the primary structure of three distinct subtypes of natriuretic peptide receptors (NPRs), which are currently designated as NPR-A and, NPR-B, and NPR-C [1]. ANP and BNP show the highest binding affinity for the type A particulate guanylate cyclase-coupled receptor (GC-A, NPR-A), whereas CNP binds the type B particulate guanylate cyclase-coupled receptor (GC-B, NPR-B) [3, 4].

Most of the physiological actions of natriuretic peptides are mediated by these GC receptors through the generation of the second messenger cyclic guanosine monophosphate (cGMP). All three natriuretic peptides also bind the natriuretic peptide clearance receptor, natriuretic peptide receptor-C (NPR-C), which lacks guanylate cyclase activity primarily acting to control the local concentrations of all three natriuretic peptides through receptor mediated internalization and degradation [5, 6].

Autoradiography has identified ANP in the Type II alveolar epithelial cells of rat lung, and NPR-A is expressed in various tissues including lung [7-9], suggesting the possibility that lung tissue is a target organ. In effect, ANP relaxes guinea pig [10, 11], rat [10], bovine [12] and human [13] airway smooth muscle *in vitro*. 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 histamine [16], propranolol [17], and nebulized water [18].

BNP shows a potent binding affinity for NPR-A [3, 4]. This finding suggests that BNP might have a role on airway smooth muscle in the same manner as does ANP. In effect, BNP relaxes guinea pig tracheal smooth muscle *in vitro* [19] and is effective in preventing ovalbumin-induced bronchoconstriction and microvascular leakage in guinea pigs *in vivo* [20]. In spite of these facts and

the documentation that BNP levels are elevated at least in patients with pulmonary disease and right ventricular dysfunction [21], published studies on BNP in human airways *in vitro* are still lacking in the literature.

Therefore, the aim of this study was to investigate the effects of BNP on human isolated bronchi constriction induced by carbachol and histamine. Considering the efficacy of BNP in preventing ovalbumin-induced bronchoconstriction in guinea pigs [20], we also tested the effect of BNP in passively sensitized human airways *in vitro*.

## Materials and Methods

### *Tissue Preparations*

Macroscopically normal airways, taken from an area as far as possible from the malignancy and dissected free of parenchyma, were obtained from 6 patients undergoing surgery for lung cancer but without a history of chronic airway disease. They were immediately placed into oxygenated Krebs-Henseleit buffer solution (NaCl, 119.0 mM; KCl, 5.4 mM; CaCl<sub>2</sub>, 2.5 mM; KH<sub>2</sub>PO<sub>4</sub> mM, 1.2 mM; MgSO<sub>4</sub>, 1.2 mM; NaHCO<sub>3</sub>, 25.0 mM; glucose, 11.7 mM) containing the cyclooxygenase inhibitor indomethacin, (5.0 μM), and transported to the laboratory. None of the patients were chronically treated with theophylline, β<sub>2</sub>-adrenoceptor agonists, corticosteroids or anticholinergic drugs. Preoperative lung function parameters were generally normal. Serum IgE levels on the day of surgery were determined for all patients to ensure that the tissues had not been sensitized prior to our interventions; they were always in the normal range. Samples were rotated overnight at room temperature in tubes containing Krebs-Henseleit buffer solution in the presence of 10% vol<sup>-1</sup> serum from healthy and non-asthmatic patients with serum IgE levels in the normal range (nonsensitized rings) or in the presence of 10% vol<sup>-1</sup> sensitizing serum from asthmatic patients (passively sensitized rings). The sensitizing serum was prepared from the whole blood of patients suffering from atopic asthma (total IgE >250 u ml<sup>-1</sup>, and specific IgE antibodies against common aeroallergens) during an exacerbation. Passive sensitization of human airway smooth muscle has been described previously [22]. Sera were not pooled but frozen at -80°C

in 200-ml aliquots until required. The next morning, after removal of adhering fat and connective tissues, bronchial rings with intact epithelium were transferred into 10 ml organ baths containing Krebs-Henseleit buffer (37°C) and continuously aerated with a 95:5% mixture of O<sub>2</sub>:CO<sub>2</sub>.

#### *Tension measurements*

Bronchial rings were mounted on hooks where one hook was attached with thread to a stationary rod and the other hook tied with thread to an isometric force displacement transducer. Bronchial rings were allowed to equilibrate for 90 min containing modified Krebs-Henseleit buffer, which was changed every 10 min. During equilibration passive tension (between 0.5 and 1.0 gram) was determined by gentle stretching of tissue. The isometric change in tension was measured with a transducer Fort 10 WPI (Basile, Instruments, Italy).

The tissue responsiveness was assessed by acetylcholine 100 µM. When the response reached a plateau, rings were washed three times and allowed to equilibrate for 30 min. Then concentration-response curves to carbachol or histamine were constructed. The submaximal response (approximately 70% maximum response, EC<sub>70</sub>) to carbachol and histamine was established for each agonist. The relaxant effect of BNP was then assessed in all rings contracted with carbachol and histamine at the sub maximal concentration (EC<sub>70</sub>) (table 1) and allowed a 15-min stabilization period, after which cumulative-concentration-response curves were constructed to BNP ranging from 1nM to 10µM (figure 1). Each concentration-response curve was obtained by the cumulative addition of BNP at intervals of 5-15 min to reach a stable level of relaxation before the next addition was made. At the end of the experiments papaverine (500 µM) was added to the bronchial rings to determine the maximal relaxant response achievable for each isolated bronchi (figure 1).

#### *Analysis*

Appropriate curve-fitting to a sigmoidal model was used to calculate the half maximal effective concentration (EC<sub>50</sub>) and the maximal response (E<sub>max</sub>) values. In the figures, the relaxant responses were expressed as percentage of papaverine (500 µM) induced relaxation, and all values are presented as mean

± SD. Statistical significance was assessed by multifactorial analysis of variance (ANOVA), with Dunnet's multiple comparison test. The level of statistical significance was defined as  $P \leq 0.05$ . All data analysis was performed using computer software (GraphPad Prism, CA, USA).

### *Drugs*

Acetylcholine, carbachol, histamine and BNP were obtained from Sigma (St. Louis, USA). All drugs were dissolved in KH solution.

## **Results**

### *Baseline characteristics of the bronchial rings*

There was no significant difference ( $P > 0.05$ ) neither in nonsensitized, nor in passively sensitized bronchi, in wet weight (mg) evaluated before the start of experiments (nonsensitized and carbachol  $169 \pm 7.87$ ; sensitized and carbachol:  $171 \pm 9.19$ ; nonsensitized and histamine:  $166 \pm 7.28$ ; sensitized and histamine,  $170 \pm 8.93$ ) or in the contraction (g) induced by acetylcholine  $100 \mu\text{M}$  (nonsensitized and carbachol:  $0.97 \pm 0.09$ ; sensitized and carbachol:  $0.94 \pm 0.20$ ; nonsensitized and histamine:  $0.92 \pm 0.11$ ; sensitized and histamine:  $0.95 \pm 0.13$ ).

### *Effect of carbachol and histamine on isolated bronchial rings*

Carbachol induced a concentration-dependent contraction without statistical differences in the  $E_{\text{max}}$  or  $EC_{70}$  in each group of bronchi (sensitized and nonsensitized) (table 1). Histamine induced a concentration-dependent contraction significantly different ( $P < 0.05$ ) in the  $E_{\text{max}}$  and  $EC_{70}$  between nonsensitized and sensitized group (table 1).

### *Relaxant effect of BNP on carbachol-contracted bronchi*

BNP induced a weak relaxant effect ( $< 30\%$ ) on carbachol-contracted bronchi in nonsensitized preparations ( $E_{\text{max}}$ , %:  $4.23 \pm 0.51$ ;  $pD_2$ :  $6.84 \pm 0.15$ ), in comparison to the relaxant effects showed by papaverine (table 2; figure 2). The effect of BNP was significantly more evident on carbachol-contracted passively sensitized bronchi ( $P < 0.05$ ) ( $E_{\text{max}}$ , %:  $11.31 \pm 2.22$ ;  $pD_2$ :



7.00±0.22) than on nonsensitized samples, but it was weak (<30%) when compared with that induced by papaverine (table 2). The BNP dose response curve was significantly ( $P<0.001$ ) shifted down in sensitized bronchi respect to nonsensitized treatments (figure 2).

#### *Relaxant effect of BNP on histamine-contracted bronchi*

BNP induced a relaxant activity on histamine-contracted bronchi in nonsensitized preparations ( $E_{max}$ , %: 42.52±9.03;  $pD_2$ : 6.33±0.06) (table 3; figure 3). The effect of BNP on histamine-contracted passively sensitized bronchi was significantly ( $P<0.05$ ) more evident than in nonsensitized samples ( $E_{max}$ , %: 60.57±9.58;  $pD_2$ : 60.57±9.58) (table 3). The BNP dose response curve was significantly ( $P$  from <0.05 to <0.001, depending on the BNP concentration) shifted down in sensitized bronchi respect to nonsensitized treatments (figure 3).

#### **Discussion**

The ANP level is significantly increased during severe asthma attacks, suggesting that ANP might have important bronchoprotective effects in asthma [23]. Also BNP levels are elevated in patients with pulmonary disease, at least in those with concomitant right ventricular dysfunction [21]. Moreover, elevated BNP concentrations resulting from pulmonary hypertension secondary to end-stage lung disease have been reported [24] even in the absence of left ventricular failure [25]. Unfortunately, the pathophysiologic consequences of these elevated concentrations are not completely understood, although there is a growing, but still poor and sometimes contrasting, evidence documenting that natriuretic peptide hormones play an important role in several biologic pulmonary activities, such as vasodilatation, bronchorelaxation, pulmonary permeability, and surfactant production and action [26]. In particular, there is documentation that ANP produces significant bronchodilation when given intravenously to both normal [27] and asthmatic subjects [13] and by inhalation in high doses in asthmatics [28]. Moreover, recently it has been documented that human recombinant BNP (nesiritide) is a potent bronchodilator in asthmatic patients [29].

Our results demonstrate that, at least in human isolated bronchi, BNP is ineffective in relaxing smooth muscle contracted by carbachol either in nonsensitized and sensitized preparations, whereas it shows a weak relaxant activity in sensitized bronchial rings contracted by histamine. This finding contrasts with the documentation that BNP elicits a potent bronchodilator effect in guinea pigs [19, 20]. Nonetheless, it must be mentioned that results from animal models cannot necessarily be applied to humans since BNP structures are highly diverse among species [1].

It is difficult to explain the discrepancy between our *in vitro* data and the *in vivo* effects observed in asthmatic patients. We completely agree with Candenas et al. [30] and Labat et al. [31], who suggested that there are no receptors for BNP on human airway smooth muscle and, consequently the bronchodilator effect seen was an indirect one. In effect, specific receptors for BNP have not been sought directly in human lung, although Hamad et al. [32], using pharmacological tools to characterize the presence of these receptors in cultured human airway smooth muscle cells, showed that treatment of these preparations with ANP, BNP, and CNP caused time- and concentration-dependent increases in cGMP level.

We must highlight that we have used isolated bronchi from non atopic patients undergoing surgery because of lung cancer, whereas human recombinant BNP (nesiritide) is a potent bronchodilator in asthmatic patients [29]. These findings fit with the observation of Ohbayashi et al. [20], who reported that ANP and BNP failed to relax guinea pig airways but both peptides inhibited antigen-induced bronchoconstriction in sensitized animals, suggesting that natriuretic peptide receptors might be expressed on airway smooth muscle during an inflammatory process.

For this reason, we have also explored if the effects of BNP might be modified by airway passive sensitization. In effect, we observed that BNP was more effective in passively sensitized airways contracted with histamine when compared with nonsensitized airways, although the broncholytic effect was weak (about 50%) when compared with that induced by papaverine. It must be mentioned that the *in vitro* model of passively sensitized human airways,

i.e. the incubation of isolated airways with IgE-rich serum obtained from atopic individuals, closely mimics features of bronchial hyperresponsiveness [22, 33], a central abnormality in patients with asthma. It has been suggested that passive immune sensitization of human tissue confers an inherent change in the contractility of airway smooth muscle that may contribute to hyperresponsiveness, which could induce mast cell degranulation [33].

In our opinion, the discrepancy between the lack of a true broncholytic activity of BNP in passively sensitized airways that we have observed and the documentation that IV human recombinant BNP is an effective bronchodilator in patients with asthma can be justified by the fact that BNP does not act as a true bronchodilator and it is likely that the effect recorded in asthmatic subjects must be linked to different actions than the simple bronchodilation. As correctly stressed by Ohbayashi et al. [20], airway microvascular leakage followed by airway inflammation and plasma exudation into the airway is one of the most difficult factors complicating asthmatic conditions [34, 35]. Therefore, the reduction of microvascular leakage with plasma exudation into the airway may be a more valuable antiasthmatic strategy than the bronchodilator effect. In effect, there is documentation that natriuretic peptide hormones elicit significant potent inhibitory effects on antigen-induced microvascular leakage [20]. Alternatively, one could postulate that this discrepancy may depend on the activation of natriuretic peptide receptors that are not located on airway smooth muscle and, consequently, the observed effect might be due to an indirect consequence of BNP. In effect, natriuretic peptide-release of NO by other lung cells, such as macrophages and epithelial cells may play a role in bronchodilation [36].

In conclusion this study suggests that BNP only induces a modest relaxant effect on human airway smooth muscle, although we believe that further studies are needed to investigate its real role in asthma and, likely, COPD.

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**Table 1.** Contracturant effects of carbachol and histamine on human isolated bronchi. Emax represents the maximal tension produced by addition of carbachol or histamine. Values are means  $\pm$  SD. \*  $P \leq 0.05$ ; significantly differences are between sensitized and nonsensitized treatments.

	Nonsensitized bronchi		Sensitized bronchi	
	Emax g	EC <sub>70</sub> $\mu$ M	Emax	EC <sub>70</sub> $\mu$ M
<b>Carbachol</b>	2.12 $\pm$ 0.44	9.3 $\pm$ 6.10	2.09 $\pm$ 0.53	9.35 $\pm$ 7.48
<b>Histamine</b>	0.95 $\pm$ 0.22	27.5 $\pm$ 5.00	*1.38 $\pm$ 0.06	*9.88 $\pm$ 1.25

C, nonsensitized bronchi; S, passively sensitized bronchi

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**Table 2.** Relaxant effects of BNP and papaverine on human isolated bronchi contracted by carbachol. Emax represents the maximal relaxation produced by addition of BNP or papaverine. Values are means  $\pm$  SD. \*  $P \leq 0.05$ ; significantly differences are between sensitized and nonsensitized treatments.

	BNP			Papaverine
	Emax (g)	% vs Papaverine	pD2	Emax (g)
<b>C</b>	0.1 $\pm$ 0,01	4.23 $\pm$ 0.51	6.84 $\pm$ 0.15	2.46 $\pm$ 0.62
<b>S</b>	*0.29 $\pm$ 0.06	*11.31 $\pm$ 2.22	7.00 $\pm$ 0.22	2.60 $\pm$ 0.69

C, nonsensitized bronchi; S, passively sensitized bronchi

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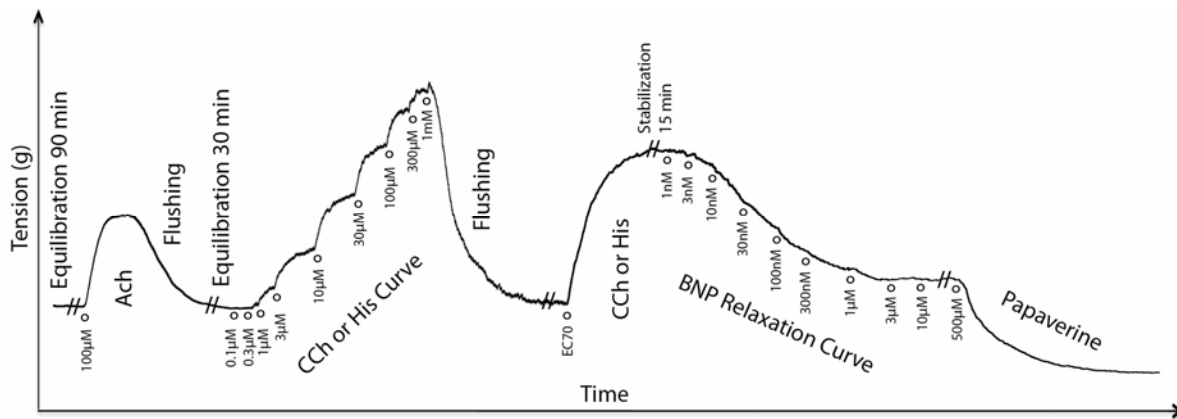
**Table 3.** Relaxant effects of BNP and papaverine on human isolated bronchi contracted by histamine. Emax represents the maximal relaxation produced by addition of BNP or papaverine. Values are means  $\pm$  SD. \*  $P \leq 0.05$ ; significantly differences are between sensitized and nonsensitized treatments.

	BNP			Papaverine
	Emax (g)	% vs Papaverine	pD2	Emax (g)
<b>C</b>	0.49 $\pm$ 0.10	42.52 $\pm$ 9.03	6.33 $\pm$ 0.06	1.15 $\pm$ 0.47
<b>S</b>	*1.20 $\pm$ 0.19	*60.57 $\pm$ 9.58	6.19 $\pm$ 0.11	1.98 $\pm$ 0.73

C, nonsensitized bronchi; S, passively sensitized bronchi

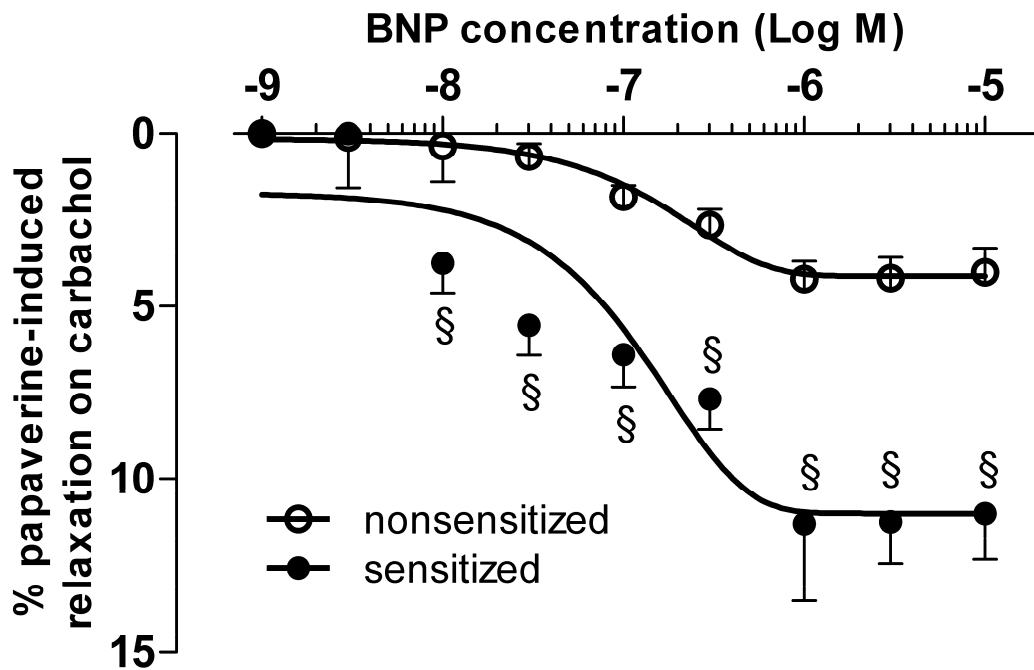
ACCEPTED MANUSCRIPT

**Figure 1.** Example of raw data from a typical experiment on BNP and isolated bronchi. Ach: acetylcholine, CCh: carbachol, His: histamine; // indicate a time contraction.



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**Figure 2.** Concentration-dependent effect of BNP on carbachol-induced contraction at a sub maximal concentration ( $EC_{70}$ ) compared with that elicited by papaverine 500  $\mu$ M in nonsensitized and passively sensitized bronchi contracted by carbachol. Each point represents mean  $\pm$  SD of 6 tissues. §  $P \leq 0.001$ ; significantly differences are between sensitized and nonsensitized treatments.



**Figure 3.** Concentration-dependent effect of BNP on histamine-induced contraction at a sub maximal concentration ( $EC_{70}$ ) compared with that elicited by papaverine 500  $\mu$ M in nonsensitized and passively sensitized bronchi contracted by histamine (His). Each point represents mean  $\pm$  SD of 6 tissues. \*  $P \leq 0.05$ ; #  $P \leq 0.01$ ; §  $P \leq 0.001$ ; significantly differences are between sensitized and nonsensitized treatments.

