Urinary Levels of Bombesin-like Peptides in Asymptomatic Cigarette Smokers: A Potential Risk Marker for Smoking-related Diseases

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Abstract

Bombesin-like peptides (BLP) produced by pulmonary neuroendocrine cells have many physiological actions which are relevant to the pathobiology of cigarette smoking. The objectives of this study were to determine whether cigarette smokers excrete increased levels of BLP in their urine compared with nonsmokers, to determine the relationship between BLP levels in urine and bronchoalveolar lavage (BAL) fluid, and whether urinary BLP levels are merely a reflection of exposure to cigarette smoke. Simultaneous BAL fluid and urine samples were obtained from ten clinically normal smokers and 22 normal nonsmoker volunteers. Urine samples were also obtained from 39 normal smokers and 30 normal nonsmokers who did not have BAL performed. BLP levels were measured in urine and BAL fluid using an enzyme-linked immunoassay. Exhaled air content of carbon monoxide, which reflects recent exposure to cigarette smoke, was determined in 34 of the clinically normal smokers and compared with urinary BLP levels. We found that, in addition to having increased BLP levels in BAL fluid (P = 0.04), asymptomatic cigarette smokers also have increased BLP levels in their urine compared with normal nonsmokers (P = 0.007). Of note, a subgroup of smokers have markedly increased BLP levels which do not overlap with the nonsmokers. Urinary BLP levels correlated with expired air content of carbon monoxide (r = 0.49, P < 0.01). However, not all smokers with increased expired air content of carbon monoxide exhibited increased BLP levels. Finally, all smokers with detectable BLP levels in BAL fluid had detectable urinary BLP levels, and there was a positive correlation between BLP levels in urine and BAL fluid (r = 0.625, P < 0.001). We conclude that a subgroup of asymptomatic cigarette smokers exhibit increased BLP levels, measurable in both urine and BAL fluid, which precede the onset of clinically detectable disease and which are not strictly dependent on smoking intensity. We speculate that smokers with increased BLP levels may have a greater risk for smoking-related diseases.

Introduction

BLP are neuropeptides produced by pulmonary NE cells that have many pharmacological actions relevant to the pathobiology of cigarette smoking (1–11). We have reported that some asymptomatic cigarette smokers without clinically detectable disease have increased BLP levels in their BAL fluid (12). This suggests that increased BLP levels precede the onset of clinical problems associated with smoking and, because of the aforementioned BLP effects, evokes the possibility of NE cells and BLP having a pathogenic role in these disorders. In addition, although there is no evidence that smokers with increased BLP levels have an increased risk for developing smoking-related diseases, recent investigations suggest that pulmonary NE cells with BLP immunoreactivity are increased in patients with tobacco-associated lung diseases (13–15), compared with normal smokers without lung disease.

In order to test the hypothesis that persistently high levels of BLP are associated with development of smoking-related disorders, a prospective evaluation of a large cohort of cigarette smokers seems a reasonable project. Unfortunately, BAL is an invasive and expensive procedure, not optimal for screening large populations during cross-sectional and prospective studies, or to assess the effect of interventions such as smoking cessation on BLP levels. Therefore, we sought to determine whether, in addition to having increased BAL fluid BLP levels, asymptomatic cigarette smokers also excrete increased levels of BLP in their urine and whether there is a correlation between BAL fluid and urinary levels of BLP. Finally, because there is some correlation between greater cigarette consumption and risk for developing lung disease, we investigated the possibility that urinary BLP levels reflect intensity of exposure to cigarette smoke.

Materials and Methods

Subjects. We measured BLP levels in urine in 101 normal, asymptomatic subjects: 52 nonsmokers and 49 current smokers. In addition, BLP levels were measured in both the BAL fluid and urine from 22 of these normal nonsmokers and 10 normal smokers who were of a similar age group and who volunteered for the procedure. None of these subjects were included in our previous study of BAL fluid (12).

Informed consent was obtained from each subject, and the protocol was approved by the Institutional Human Subjects Review Committee. A detailed smoking history was obtained from each subject, and the cumulative cigarette consumption was expressed in packs/years. All of these subjects denied any major respiratory symptoms (persistent cough, dyspnea) and had normal physical examinations. In addition, those subjects undergoing bronchoscopy and BAL had normal chest radiographs, spirometric test results, lung volumes, and diffusing capacity for carbon monoxide.

The abbreviations used are: BLP, bombesin-like peptides; BAL, bronchoalveolar lavage; GRP, gastrin-releasing peptide; NE, neuroendocrine; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; ELISA, enzyme-linked immunosorbent assay.


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Expired CO. Expired air content of carbon monoxide, a reflection of recent exposure to cigarette smoke which correlates with levels of carboxyhemoglobin (16), was determined with a portable carbon monoxide analyzer (Ecoyzer; Analysis Automation, Ltd.).

Urinary Samples. All urine samples (10 ml) were collected in the morning, after emptying the bladder of the overnight specimens; immediately centrifuged for 10 min; and divided into two 5-ml aliquots, one for creatinine determination and the other for BLP analysis. The sample for BLP measurement was immediately preserved by the addition of acetic acid to a final concentration of 2 N and stored at -70°C. Twenty-four h prior to assay, 2-ml aliquots of urine were vacuum dried in a Savant Speed Vac concentrator (Savant Instruments, Farmingdale, NY) and reconstituted to 25% of the original volume with 0.05 M HEPES buffer, pH 7.0, in HPLC-grade water.

BAL Fluid. BAL was performed as previously described (12). Briefly, BAL fluid was collected after sequential instillation of four 60-ml aliquots of sterile normal saline solution through a fiberoptic bronchoscope and immediately placed in ice water. The lavage was centrifuged at 800 x g for 10 min. Cell-free BAL fluid supernatants were preserved in a final concentration of 2 N acetic acid and stored at -70°C. Twenty-four h prior to assay, 2-ml aliquots of BAL fluid were vacuum dried and reconstituted to 25% of the original volume with 0.05 M HEPES buffer, pH 7.0, in HPLC-grade water.

BLP Immunooassay. BLP is the name given to all peptides having carboxy-terminal homology with the structure of bombesin, a tetrapeptide originally isolated by Erspamer et al. (17) from the skin of the amphibian Bombina bombina. This family of neuropeptides includes GRP and neuromedin B, the only human BLP characterized to date (18). We use the term BLP for the immunoreactive peptides detected by our ELISA because the monoclonal antibody BBC353 is specific for the carboxy terminal shared by all known BLP, whether oxidized or reduced, but cannot distinguish between their species (12). Nonetheless, the specificity of immune staining that human lung NE cells demonstrate with the antibody BBC353 supports its utility for these studies as it suggests that we are measuring NE cell-derived BLP (12, 15).

Briefly, Nunc-Immuno I testing plates (InterMed, Denmark), were coated with lys-bombesin (50 ng/well; Peninsula) and blocked with 0.05% Tween 20 in PBS for 2 h at 4°C. Lys-bombesin was found to bind more reproducibly in the plates than bombesin. Use of blocking solutions containing albumin at this stage greatly reduces the sensitivity of the assay. After washing twice with PBS, standard concentrations of bombesin (Peninsula) and unknown BAL fluid and urine samples (50 µl/well) were added to the wells and incubated for 1 h at room temperature with the anti-bombesin monoclonal antibody BBC353 (50 µl of a 50-ng/ml solution). Unbound BBC353 was removed by washing twice with PBS, and peroxidase-labeled goat antimouse antibody was removed by washing twice. The chromogenic substrate 2,2'-azinobis-3-ethylbenzthiazoline sulfonic acid was added in 0.1 M buffer, pH 7.0, in HPLC-grade water.

Fig. 1. In vitro degradation rates of bombesin by human plasma, urine, and BAL fluid (BALF). Points, mean; bars, SE.

Results

In Vitro Degradation of BLP. The in vitro degradation of bombesin at 37°C was relatively rapid in plasma (Fig. 1). Within the first 20 min, bombesin immunoreactivity decreased by almost 60% and decreased to 20% of the initial immunoreactivity after 300 min. In comparison, disappearance of bombesin immunoreactivity from urine and BAL fluid samples from the same subjects was much slower, and more than 75% of the initial bombesin immunoreactivity was still detectable after 300-min incubation. GRP and neuromedin B, which share a highly homologous carboxy-terminal fragment detected by this BLP ELISA, exhibited similar degradation rates (data not shown).

Urinary Levels of BLP. Measurable urine levels of BLP (above 0.2 pmol/ml/mg of creatinine) were found in 46 of the 49 asymptomatic cigarette smokers, but only in 34 of the 52 normal nonsmokers. After normalizing each result to the urinary creatinine content, the smokers demonstrated measurable levels ranging from 0.28 to 53.85 pmol/mg of creatinine. In the nonsmokers, however, measurable levels ranged from 0.21 to 8.80 pmol/mg of creatinine. Assigning the minimal detectable value of the ELISA (0.2 pmol/ml/mg of creatinine) to the negative samples for statistical purposes, the asymptomatic cigarette smokers exhibited a mean value of 5.12 ± 1.2 pmol/mg of creatinine. Meanwhile, the normal nonsmokers exhibited a mean value of 1.81 ± 0.32 pmol/mg of creatinine, which was significantly lower (P = 0.0707, Student's t test; Fig. 2) compared with the smokers. Statistical analysis of these results using the median test, in order to avoid assigning an arbitrary value to the undetectable samples, also demonstrated a difference (P < 0.001). Moreover, a significant proportion (7 of 49, 14% of the asymptomatic smokers) of the cigarette smokers exhibited undetectable levels of BLP, whereas 2 of 52 (4%) of the normal nonsmokers exhibited undetectable levels (P = 0.007, Student's t test; Fig. 2).
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![Graph 1: BAL Fluid BLP vs. Urine BLP](image1)

**Fig. 3.** BAL fluid levels of BLP in asymptomatic cigarette smokers (n = 10) and normal nonsmokers (n = 22).

![Graph 2: correlations between BAL Fluid BLP and Urine BLP](image2)

**Fig. 4.** Correlation between urinary and BAL fluid levels of BLP. Overlapping points are denoted by numbers above them in the graph. BALF, BAL fluid; créât, creatinine.

![Graph 3: Correlation between Urine BLP and Expired Air CO](image3)

**Fig. 5.** Correlation between urinary levels of BLP and expired air content of carbon monoxide. Overlapping points are denoted by numbers above them in the graph. creat, creatinine.

14% of smokers had urinary BLP levels in excess of 10 pmol/mg of creatinine, but none of the nonsmokers had levels in this range.

**BAL Fluid Levels of BLP.** Measurable BAL fluid levels of BLP were found in 8 of 10 asymptomatic cigarette smokers and 7 of 22 normal nonsmokers (Fig. 3). In the smokers, measurable BLP levels ranged from 0.5 to 28.2 pmol/ml. After assigning the minimal detectable value of the ELISA (0.2 pmol/ml) to the negative samples for statistical purposes, the asymptomatic cigarette smokers demonstrated a mean value of 4.58 ± 2.89 pmol/ml compared with 0.53 ± 0.12 pmol/ml for the normal nonsmokers (P = 0.04, Student's t test; P = 0.01, median test). In the subgroup of subjects who underwent BAL, the urinary levels of BLP were also higher in the asymptomatic cigarette smokers (4.33 ± 1.33 pmol/mg of creatinine) than in the normal nonsmokers (2.43 ± 0.61 pmol/mg of creatinine), but this difference was not statistically significant (P = 0.143, Student's t test; P = 0.8, median test).

**Correlations with Urinary Levels of BLP.** All smokers with detectable BLP levels in BAL fluid had detectable urinary BLP levels, and there was a statistically significant correlation between urinary and BAL fluid levels of BLP (r = 0.625, P < 0.001, Spearman rank correlation coefficient). Of note, 2 of the 10 smokers and 7 of the 22 nonsmokers had detectable BLP levels in urine but not in their BAL fluid (Fig. 4), which suggests the possibility of extrapulmonary BLP sources. As previously demonstrated with BAL fluid levels of BLP (12), there was no correlation between urinary BAL levels and cumulative cigarette consumption expressed in packs/years. In addition, in the 34 smokers whose expired air content of carbon monoxide was determined, there was a positive correlation (r = 0.45, P < 0.01, Spearman rank correlation coefficient: Fig. 5) between expired air content of carbon monoxide and urinary BLP levels. However, not all smokers with increased expired air content of carbon monoxide exhibited increased urinary BLP levels despite this positive correlation. Therefore, urinary BLP levels do not merely reflect intensity of exposure to cigarette smoke, whether the latter is determined by smoking history or expired air content of carbon monoxide.

**Discussion**

Cigarette smoking has significant effects on the pulmonary NE cells (12–15, 20–24). The study of NE cell-derived peptides, such as calcitonin and BLP, has made this relationship more readily apparent. Becker and coworkers have demonstrated immunoreactive calcitonin within the pulmonary NE cell (25) as well as the stimulatory effect of cigarette smoke on calcitonin secretion from these cells (26). In addition, they have measured increased levels of calcitonin in the serum and urine of patients with a variety of lung diseases, including smokers with emphysema (27). We have chosen BLP as a marker for NE cell stimulation by cigarette smoke because their physiological effects, such as being a growth factor (7), stimulating mucous secretions (8), regulating gastric acid secretion (9), and affecting food intake (10, 11), seem more obviously relevant to the pathophysiology of cigarette smoking than what we know to date about calcitonin and other NE cell-derived mediators.

The potential relevance of NE cell-derived BLP to smoking-related diseases is supported by observations from Gosney et al. who have quantitated NE cells with BLP immunoreactivity in normal adult lungs (28) and demonstrated a 2-fold increase of these cells in smokers with chronic bronchitis and emphysema (14). In addition, we have made the observation that adult patients with eosinophilic granuloma, a disease associated with cigarette smoking, have increased pulmonary NE cells with BLP immunoreactivity compared with normal smokers without lung disease (15). Finally, we have also demonstrated that some asymptomatic cigarette smokers have increased BLP levels in their BAL fluid before the onset of clinically detectable disease (12), while Tabassian et al. (24) have demonstrated in an animal model that increased BLP production may occur within weeks of exposure to cigarette smoke. All of these observations suggest that smoking-related diseases are associated with increased numbers of NE cells which produce BLP and that these abnormalities of NE cells in cigarette smokers occur as an early event which may potentially contribute to the pathogenesis of smoking-related diseases. Thus, it seems attractive to consider longitudinal prospective studies on BLP levels as a potential risk indicator for smoking-related diseases.

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Given the invasive nature of the BAL procedure, we designed this study to assess the role of urinary BLP level measurements in cigarette smokers. Plasma BLP measurements, although less invasive than BAL, are not practical because the reported values in both healthy and diseased subjects are below the sensitivity of our immunoassay (29, 30). Since BLP are degraded much faster by human plasma than by urine or BAL fluid (Fig. 1), this may contribute to the lower BLP levels detected in plasma. Of note, the urinary BLP levels reported in this study are consistent with the plasma levels of GRP immunoreactivity reported in human subjects by Haraguchi et al. (30), assuming normal renal function. These investigators found that patients with chronic renal failure exhibited predialysis plasma levels of GRP immunoreactivity that were significantly higher than those in healthy controls. This observation, in addition to previously reported data on calcitonin (27), supports urinary excretion as a major clearance route for neuropeptides.

Using this ELISA for BLP immunoreactivity, we cannot distinguish between specific forms of BLP such as GRP and neuromedin B, or perhaps novel forms of BLP which are yet to be characterized but could have a homologous carboxy-terminal fragment. In addition, both urinary and BAL fluid measurements of BLP levels exhibit a great variability between subjects. This may result from individual variations in the handling of cigarette smoke as well as variations in neuropeptide degradation mechanisms, such as neutral endopeptidase activity, which could also be affected by cigarette smoke (31). Nonetheless, in spite of this variability and the fact that BLP in BAL fluid and urine remain to be fully characterized, we consistently found that a subgroup of asymptomatic cigarette smokers not only have increased BLP levels in their BAL fluid (12), but also excrete increased levels of BLP in their urine when compared with normal nonsmokers. Furthermore, there is a correlation between BAL fluid and urinary levels of BLP despite the various subjects in whom there was undetectable BLP immunoreactivity in BAL fluid but increased urinary BLP levels. The latter may suggest that other body sources of BLP, besides the pulmonary NE cells, could be important contributors to BLP levels measured in urine. This would further emphasize that cigarette smoke affects the lungs as well as the whole organism. Importantly, there were no subjects in our study with increased BLP levels in BAL fluid and undetectable levels in urine (Fig. 4). Thus, urinary measurements of BLP levels may prove useful as a sensitive screening test, albeit not entirely specific, for susceptibility of the pulmonary NE cells to the effects of cigarette smoke.

Finally, there is also a great variability in different individuals’ susceptibility to the deleterious effects of cigarette smoke. Although there is a direct relationship between greater cigarette consumption and deterioration of lung function (2), only a minority (10 to 15%) of smokers develop clinically significant obstructive disease, even among heavy smokers (2, 32–35). The reasons for the increased susceptibility in this subgroup of smokers remain unknown; however, it appears that these susceptible smokers with airflow obstruction are also those at greater risk for developing lung cancer (36, 37). Of note, urinary BLP levels in asymptomatic cigarette smokers also tend to reflect smoking intensity as determined by expired air content of carbon monoxide, but only a minority of smokers with increased expired air content of carbon monoxide exhibit markedly increased BLP levels which do not overlap with those of the nonsmokers. We speculate that this subgroup of smokers (~15%) who exhibit markedly increased BLP levels in both BAL fluid and urine may be the group at risk for developing tobacco-associated diseases, which may or may not result from abnormalities of the NE cells. Further investigations may establish a definite role for NE cells and BLP in the pathogenesis of tobacco-associated disorders.

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References

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