

cAMP and cGMP in nasal mucus related to severity of smell loss in patients with smell dysfunction

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Abstract

Purpose: To evaluate nasal mucus levels of cAMP and cGMP in patients with taste and smell dysfunction with respect to severity of their smell loss.

Methods: cAMP and cGMP were measured in nasal mucus using a sensitive spectrophotometric 96 plate ELISA technique. Smell loss was measured in patients with taste and smell dysfunction by standardized psychophysical measurements of olfactory function and classified by severity of loss into four types from most severe to least severe such that anosmia > Type I hyposmia > Type II hyposmia > Type III hyposmia. Measurements of nasal mucus cyclic nucleotides and smell loss were made independently.

Results: As smell loss severity increased stepwise cAMP and cGMP levels decreased stepwise [cAMP, cGMP (in pmol/ml); anosmia – 0.004, 0.008; Type I hyposmia – 0.12±0.03, 0.10±0.03; Type II hyposmia – 0.15±0.02, 0.16±0.01; Type III hyposmia – 0.23±0.05, 0.20±0.15].

Conclusions: These results confirm the association of biochemical changes in cyclic nucleotides with systematic losses of smell acuity. These results confirm the usefulness of the psychophysical methods we defined to determine the systematic classification of smell loss severity. These changes can form the basis for the biochemical definition of smell loss among some patients with smell loss as well as for their therapy.

We have previously reported that saliva levels of both cAMP and cGMP were lower in patients with taste and smell dysfunction than in normal subjects.¹ We have also published preliminary studies that nasal mucus levels of both cAMP and cGMP in patients with taste and smell dysfunction were lower than in normal subjects.^{2,3} Using these data, we suggested that lower than normal levels of nasal cyclic nucleotides may play a role in clinical induction of loss of smell.^{2,3} In order to learn more about this system we hypothesized that levels of nasal mucus cyclic nucleotides in patients with smell loss might correlate with their degree of smell loss. It was possible to perform this type of study since we had previously categorized smell loss among these patients into distinct pathophysiological categories, using a systematic, standardized psychophysical process.⁴⁻⁷ These psychophysical categories allowed us to classify smell loss quantitatively into four types graded from most severe to least severe loss such that anosmia > hyposmia Type I > Type II > Type III. In an effort to relate these categories to biochemical parameters we compared levels of patients' nasal mucus cAMP and cGMP performed by use of a sensitive immunoassay technique in an independent manner to their severity of smell loss. Results

indicated an inverse relationship between severity of smell loss and nasal mucus cAMP and cGMP levels such that as smell loss increased (hyposmia Type III > Type II hyposmia > Type I > anosmia) nasal mucus cAMP and cGMP levels correspondingly decreased.

Methods

All studies were performed at The Taste and Smell Clinic, Washington, DC between June 2004 and November 2006 and constitute studies on consecutive normal subjects and patients. Study protocol was approved by the Institutional Review Board of the Georgetown University Medical Center. All patients participated in the study consistent with this protocol.

Nasal mucus was collected from 66 normal volunteers aged 16-79yr [53±2, Mean±SEM] without acute or chronic disease consisting of 40 men aged 19-74yr (54±3) and 26 women, aged 16-79yr (53±2). Taste and smell function was within normal limits in each subject.

Nasal mucus was also collected from 203 patients, aged 18-86yr (55±1) with taste and smell dysfunction consisting of 117 women, aged 18-85yr (55±2) and 86 men, aged 23-86yr (54±3). Patients were all patients who presented consecutively to the Taste and Smell Clinic, Washington, DC for evaluation of taste and smell dysfunction.

Nasal mucus was collected in a 50ml plastic tube over a period of one-four days using spontaneous nasal discharge. No blood was present in any sample analyzed. Amounts collected varied from 1-40ml. In the laboratory mucus was centrifuged at 20,000 rpm in a Sorvall RC 5C Plus refrigerated centrifuge for 20-50 min, the supernatant transferred to 500µl plastic tubes and stored at -20°C.

cAMP and cGMP were measured by a sensitive spectrophotometric 96 plate ELISA technique using kits supplied by R&D Systems (Minneapolis, MN). Mean variation of kit standards was ≤ 5%.^{8, 9} Protein was measured by obtaining absorbance at 215-225nm with use of the extinction coefficient by a method pre-

viously described¹⁰; in this manner protein in very small samples was estimated. cAMP and cGMP in pmol/concentration were expressed in two ways; per ml nasal mucus and per mg protein.

To determine methodological reliability cAMP and cGMP were determined in several ways. Duplicates of 17 nasal mucus samples were determined on 15 occasions; the standard deviation of these samples varied from 0.007-0.038 for cAMP and 0.007-0.038 for cGMP respectively; mean coefficients of variation varied from 1-10% for both moieties. cAMP and cGMP from one subject were determined on 17 separate occasions over a period of two years; the standard deviation for these determinations for cAMP (pmol/ml) was 0.29 with a mean coefficient of variation of 3%; for cAMP/ (pmol/mg protein), 0.13 with a mean coefficient of variation of 3%; for cAMP (pmol/ml flow rate) 0.49 with a mean coefficient of variation of 5%; for cGMP (pmol/ml), 0.02 with a coefficient of variation of 4%; for cGMP (pmol/mg protein) 0.007 with a coefficient of variation of 5%; for cGMP (pmol/ml flow rate) 0.05 with a coefficient of variation of 10%.

Smell loss was classified by psychophysical measurements of olfactory function administered to each patient through use of a standardized forced-choice, three-stimuli, step wise-staircase technique in a fixed, controlled design.^{6, 7} Efficacy of technique and results of testing were previously documented in a double-blind clinical trial.¹¹ Four odors were used which included pyridine (dead-fish odor), nitrobenzene (bitter-almond odor), thiophene (gasoline-like odor) and amyl acetate (banana-oil odor). Detection thresholds (DT), recognition thresholds (RT) and magnitude estimation (ME) values for each odor were determined as previously described.^{6, 11} Based upon the results of these tests, the degree of smell loss was classified as shown in Table 1.

Classification indicated that patients with anosmia had the greatest severity of smell loss and could neither detect nor recognize any vapor; thus DT, RT and ME were zero since no patient could detect, recognize

TABLE 1. Classification of smell loss

| | Detection threshold DT in M/L | Recognition threshold RT in M/L | Magnitude estimation MEAN ME in % |
|----------------------------------|----------------------------------|------------------------------------|--------------------------------------|
| Normals | + | +* | ≥50 |
| Patients anosmia [∞] | 0 | 0 | 0 |
| hyposmia type 1 | ± | 0 | 0 |
| type 2 | ± | ±* | <50 |

+ Normal ($\leq 10^{-5}$ M for all odorants)

+* NormalL ($\leq 10^{-2}$ M for all odorants)

0 Absent response (∞)

± Present but < normal ($> 10^{-5}$ M < ∞ for all odorants)

±* Present but < normal ($> 10^{-2}$ M < ∞ for all odorants)

∞ Inability to detect, recognize or judge intensity of an absolute concentration of odorant

or grade intensity of any odor including an absolute concentration of each odorant (Table 1). Patients with Type I hyposmia could detect some odors but could not recognize any odor correctly; thus, DTs for some odors were present but RTs and MEs for all odors were zero since no patient could recognize or thereby grade intensity of any odor (none could be recognized correctly) (Table 1). Patients with Type II hyposmia could detect and recognize some odors but at levels greater than normal; thus DTs and RTs were present but elevated above normal and MEs were present but at levels lower than normal (Table 1). Patients with Type III hyposmia could detect and recognize all odors at normal levels, i.e., normal DTs and RTs, but ME values for one or more odors were significantly decreased below normal (Table 1).

Severity of smell loss was graded from greatest to least severe loss as anosmia > hyposmia Type I > Type II > Type III (Table 1).

All studies of nasal mucus cyclic nucleotides were coded. The results of smell loss classification were obtained independent of any results of nasal mucus cyclic nucleotides. Only after all patient classifications of smell loss were completed were nasal mucus studies uncoded and related to smell loss degree.

Mean and SEM for each subject group classification were calculated. Differences between group means were calculated using Student's t tests. Results

were also analyzed by use of the Spearman rank correlation technique.

Results

Nasal mucus cAMP and cGMP in normals compared to patients categorized by smell loss severity

All patients combined had lower levels of both nasal mucus cAMP and cGMP than did normal subjects (Table 2). Categorized by smell loss severity the two patients with anosmia, the most severe form of smell loss, had the lowest levels cAMP and cGMP in nasal mucus compared to all patients combined or to patients with any type of hyposmia (Table 2). Patients with Type I hyposmia exhibited higher levels of both cAMP and cGMP than did patients with anosmia but lower than in normals, in all patients combined and in patients with Types II and III hyposmia (Table 2). Patients with Type II hyposmia had lower levels than in normals or patients with Type III hyposmia, higher levels than patients with anosmia or Type I hyposmia, but levels not significantly different from those in all patients combined (Table 2). Patients with Type III hyposmia had lower levels than in normals but levels higher than in patients with anosmia, Types I or II hyposmia.

Correlation of nasal mucus levels of cAMP and cGMP with smell loss severity using the Spearman rank order coefficient was -0.75 ($P < 0.005$) relating smell loss type to nasal mucus cAMP and -0.71 ($P < 0.005$) relating smell loss type to nasal mucus cGMP.

Nasal mucus protein did not differ among the patients although levels in each patient group were lower than in normals (Table 2).

Nasal mucus cAMP in normal men and women compared to men and women patients with smell loss

Levels of cAMP in women patients and in normal women were higher than in men patients and in normal men (Table 3). Both men with anosmia had lower

TABLE 2. cAMP and cGMP concentrations in nasal mucus in patients with anosmia and Type I and Type II hyposmia

| Patients with smell loss | cAMP | | cGMP | | Total protein mg/dl | Age yr |
|--------------------------|--------------------------|-----------------|---------------------------|---------------------------|------------------------|-----------|
| | pmol/ml | pmol/mg protein | pmol/ml | pmol/mg protein | | |
| All patients (203) | 0.15±0.02 ^{*,e} | 0.06±0.02 | 0.23±0.02 ^a | 0.10±0.01 ^b | 2.56±0.12 ^e | 54±2 |
| Anosmia (2) | 0.004 | 0.002 | 0.008 | 0.004 | 2.18 | 52 |
| Hyposmia (201) | | | | | | |
| Type I (41) | 0.12±0.03 ^b | 0.04±0.01 | 0.10±0.03 ^{a,bl} | 0.035±0.010 ^{bl} | 2.84±0.15 | 50±5 |
| Type II (151) | 0.15±0.02 ^c | 0.06±0.02 | 0.16±0.01 ^{a,bl} | 0.06±0.01 ^{a,dl} | 2.62±0.16 ^c | 51±7 |
| Type III (9) | 0.23±0.05 | 0.08±0.04 | 0.20±0.15 | 0.07±0.04 | 3.01±0.43 | 45±14 |
| Normals (66) | 0.31±0.05 | 0.12±0.04 | 0.56±0.07 | 0.19±0.03 | 3.25±0.20 | 49±7 |

() Subject Number

* Mean ± SEM

With respect to all patients

a¹ P<0.001b¹ P<0.005c¹ P<0.02d¹ P<0.05e¹ P<0.01

With respect to normals

a P<0.001

b P<0.005

c P<0.02

d P<0.05

e P<0.01

levels of both cAMP and cGMP than did normal men and women, in all patients combined and in men and women with both types of hyposmia (Table 3). Men with Type I hyposmia had lower levels of cAMP than all men patients, men patients with Type II hyposmia and normal men. Men with Type I hyposmia had lower levels of cGMP than did normal men, men with Type II hyposmia and normal men but levels were not significantly lower (Table 3). Women with Type I hyposmia had lower levels of cAMP than did normal men, women with Type II hyposmia and normal women. Both men and women with Type II hyposmia had higher levels of cAMP than did men and women with Type I hyposmia, respectively, but lower levels than in normal men and women, respectively (Table 3). Women with Type III hyposmia had lower levels of cAMP than did normal women but higher levels of both moieties than all women patients (Table 3).

Nasal mucus cGMP in normal men and women compared to men and women patients with smell loss

cGMP levels in all women patients combined, in patients with both types of hyposmia and in normal women were higher than were cAMP levels (Table 3). cGMP levels in the men with anosmia were lower than in normal men and women, and in all men and

women patients with either type of hyposmia (Table 3).

cGMP in men and women with Type I hyposmia was lower than in normal men and women, in all men and women patients combined, and in men and women with Type II hyposmia (Table 3). Men and women with Type II hyposmia had lower levels of cGMP than in normal men and women, but higher levels of cGMP than did all men and women patients combined and men and women with Type I hyposmia. Women with Type III hyposmia had lower levels of cGMP than did normal women but higher levels than did all women patients combined (Table 3).

Nasal Mucus protein

Nasal mucus protein in all men patients combined was higher than in men with any type of hyposmia but lower than in normal men (Table 3). Levels of nasal mucus protein in all women patients were lower than in normal women.

Discussion

These results indicate that smell loss severity correlates with nasal mucus levels of both cAMP and cGMP and reflect the first demonstration of this relationship among patients with loss of smell. Results of

TABLE 3. cAMP and cGMP concentrations in nasal mucus in patients with smell loss categorized by smell loss severity and gender.

| Patients with smell loss | cAMP | | cGMP | | Total protein mg/dl | Age yr |
|--------------------------|-----------------------------|-----------------|----------------------------|------------------------------|-------------------------|-----------|
| | pmol/ml | pmol/mg protein | pmol/ml | pmol/mg protein | | |
| All patients (203) | 0.15±0.02 ^{*, c} | 0.06±0.02 | 0.23±0.02 ^a | 0.10±0.01 ^b | 2.56±0.12 ^e | 54±2 |
| Men (86) | 0.14±0.04 | 0.05±0.03 | 0.20±0.03 | 0.07±0.02 | 2.77±0.17 ^{d3} | 56±2 |
| Women (117) | 0.16±0.05 ^{b1} | 0.06±0.02 | 0.23±0.02 ^{b3} | 0.09±0.01 ^{b1} | 2.60±0.08 | 54±2 |
| Anosmia (2) | 0.004 | 0.002 | 0.008 | 0.004 | 2.18 | 52 |
| men (2) | - | - | - | - | - | - |
| Hyposmia (201) | - | - | - | - | - | - |
| Type I (41) | 0.12±0.03 ^b | 0.04±0.01 | 0.10±0.03 ^{a, b1} | 0.035±0.010 ^{a, a1} | 2.84±0.15 | 48±3 |
| men (22) | 0.07±0.02 ^{a2, e4} | 0.03±0.02 | 0.09±0.05 ^{b2} | 0.03 ±0.03 | 2.61±0.29 | 50±3 |
| women (19) | 0.13±0.04 ^{b3} | 0.05±0.04 | 0.13±0.08 ^{b3} | 0.05 ±0.02 | 2.84±0.16 | 46±3 |
| Type II (151) | 0.15±0.03 ^e | 0.06±0.02 | 0.16±0.01 ^a | 0.06 ±0.01 ^{a, a1} | 2.62±0.16 ^d | 57±2 |
| men (59) | 0.15±0.02 ^{a2} | 0.05±0.02 | 0.14±0.03 ^{b2} | 0.05 ±0.02 | 2.75±0.17 ^{d2} | 58±2 |
| women (92) | 0.15±0.02 ^{b3} | 0.06±0.01 | 0.19±0.02 ^{b3} | 0.08 ±0.01 ^{b3} | 2.52±0.08 | 57±1 |
| Type III | | | | | | |
| men (3) | | | | | | |
| women (6) | 0.23±0.05 | 0.08±0.04 | 0.20±0.15 | 0.07 ±0.04 | 3.01±0.43 | 45±14 |
| Normals (66) | 0.31±0.05 | 0.12±0.04 | 0.56±0.07 | 0.19 ±0.03 | 3.25±0.20 | 49±7 |
| Men (40) | 0.23±0.002 ^{d3} | 0.07±0.04 | 0.28±0.03 ^{e3} | 0.09 ±0.03 | 3.28±0.11 | 42±18 |
| Women (26) | 0.34±0.05 | 0.13±0.06 | 0.63±0.12 | 0.20 ±0.04 | 3.15±0.43 | 49±9 |

() Subject Number

*Mean±SEM

| | | | | |
|----------------------------|------------------------------|-----------------------------|-----------------------------|------------------------------|
| With respect to normal men | With respect to normal women | With respect to all normals | With respect to Type II men | With respect to all patients |
| a2 $P<0.001$ | a3 $P<0.001$ | a $P<0.001$ | c4 $P<0.02$ | a1 $P<0.001$ |
| b2 $P<0.005$ | b3 $P<0.005$ | b $P<0.005$ | e4 $P<0.01$ | b1 $P<0.005$ |
| c2 $P<0.02$ | c3 $P<0.02$ | c $P<0.02$ | c1 $P<0.02$ | |
| d2 $P<0.05$ | d3 $P<0.05$ | d $P<0.05$ | e1 $P<0.01$ | |
| e2 $P<0.01$ | e3 $P<0.01$ | e $P<0.01$ | | |

lower than normal concentrations of these cyclic nucleotides in saliva of these same patients have been previously demonstrated.¹ Since many of the same substances in nasal mucus are also found in saliva¹² substances in both fluids may reflect similar changes in bodily function.

While previous investigators have demonstrated lower than normal levels of specific biochemical moieties in blood¹³⁻¹⁹, saliva^{20, 21} and nasal mucus²² of patients with taste and smell dysfunction, there have been few previous studies dealing with comparisons of levels of cyclic nucleotides in nasal mucus between normal subjects and patients with smell loss. There have been no prior attempts to correlate changes in any chemical moiety in any physiological fluid with functional changes in degree of smell loss.

The results present also suggest that the diagnostic methods used to classify patients with smell loss and the measurements used to measure their nasal mucus cyclic nucleotides are useful discriminatory tools by which to identify these patients.

While these patients as a group may exhibit lower than normal levels of nasal mucus cAMP and cGMP individual patients may not exhibit these lower than normal levels. Whereas all patients with anosmia or Type I hyposmia do exhibit lower than normal levels of nasal mucus cAMP some patients with Types II and III hyposmia may fall into the normal range. Thus, measurements of both olfactory acuity and nasal mucus cyclic nucleotides are necessary to identify pathology among patients who complain of smell loss.

These results may also relate to subsequent treatment for smell loss. Theophylline, a phosphodies-

terase (PDE) inhibitor, has been used successfully to treat patients with smell loss²³ who exhibit these lower than normal levels of nasal mucus cAMP and cGMP. Preliminary results indicated that treatment restored acuity in about 70% of these patients²⁴ and these results were confirmed by functional magnetic imaging studies of brain²³. Theophylline inhibits cAMP and cGMP PDE activity thereby increasing these lower than normal levels of nasal mucus cyclic nucleotides among these patients. Nasal mucus cyclic nucleotides may act to stimulate olfactory stem cell growth and maturation, as do other substances²⁵ and to inhibit cellular apoptosis.²⁶

This concept was previously observed with identification of zinc ion in evaluation and treatment of taste and smell dysfunction. Zinc deficient patients exhibit abnormalities of taste and smell function.¹³⁻¹⁷ Treatment of patients with zinc deficiency with exogenous zinc led to correction of both their zinc deficiency and their taste and smell abnormalities.¹⁷⁻²⁰ Identification of these patients with abnormalities of zinc metabolism and their zinc treatment led to an understanding of the mechanism underlying these sensory changes – i.e., their sensory losses were related to their lower than normal levels of carbonic anhydrase VI in saliva²¹ and in nasal mucus²² and their treatment with exogenous zinc corrected both abnormalities.²⁷

Nasal mucus supplies those nutrients necessary for normal maintenance of olfactory epithelial cell homeostasis.¹² The roles of cAMP and cGMP in olfactory²⁸⁻³⁰ and gustatory^{31, 32} physiology have been well known for many years. However, their roles in olfactory pathology have not been carefully explored. Present results suggest that nasal mucus cyclic nucleotides not only play roles in normal olfactory function but also in its pathology perhaps through stimulation of G protein function^{30, 33}, membrane stabilization³⁴, neuronal growth and development³⁵ and inhibition of increased apoptotic activity.³⁶

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