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Decreased parotid salivary cyclic nucleotides related to smell loss severity in patients with taste and smell dysfunction

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Abstract

Parotid salivary levels of cyclic adenosine monophosphate (cAMP) have been previously demonstrated to be lower than normal in patients with taste and smell dysfunction. To define these results more fully, we analyzed parotid salivary levels of cAMP and cyclic guanosine monophosphate (cGMP) with respect to severity of smell loss in these patients. *Smell loss severity* was defined by psychophysical measurements of olfactory function and classified into 4 types from most severe to least severe loss. This resulted in patients exhibiting, in order of loss severity (from greatest to least), anosmia > type I hyposmia > type II hyposmia > type III hyposmia. Parotid saliva cAMP and cGMP were measured independently using a sensitive spectrophotometric 96-plate enzyme-linked immunosorbent assay technique; mean levels were categorized by clinical classification of loss severity. As smell loss severity decreased, salivary cAMP and cGMP levels increased consistently with each stepwise change of clinical loss severity. This is the first demonstration of biochemical changes in saliva associated with a quantitative classification of smell loss. These results reflect a biochemical method to identify and classify patients with smell loss in some respects similar to initial typing of serum lipid levels to assist in risk classification of patients with cardiovascular disease.

1. Introduction

Many previous investigators demonstrated that cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) play roles in both taste [1,2] and smell [3-6] function. We previously reported that salivary levels of both of these moieties are lower than normal in patients with taste and smell dysfunction [7,8]. These latter results suggested that lower than normal levels of these nucleotides relate to a role these moieties play in the functional sensory pathology demonstrated by these patients [7,8]. To relate these sensory and biochemical changes to functional changes in olfactory acuity, we classified patient smell loss into 4 types by use of psychophysical measurements of smell function [9-11]. The greatest degree of smell loss was labeled *anosmia*; the next greatest, *type I hyposmia*; the next, *type II hyposmia*; and the least, *type III hyposmia* [10,11]. Independently, we measured salivary cAMP and cGMP in each patient [8]. We then compared measurements of salivary cyclic nucleotide with respect to each smell loss classification. These results indicated an inverse relationship between severity of smell loss and salivary cAMP and cGMP levels; as smell loss acuity decreased (anosmia > type I hyposmia > type II hyposmia > type III hyposmia), salivary cAMP and cGMP levels correspondingly increased. We have previously demonstrated relationships between both smell loss and nasal mucus cyclic nucleotides [12] and smell loss severity and nasal mucus cyclic nucleotides [13]; we now demonstrate this latter relationship with salivary cyclic nucleotides.

2. Methods

All studies were performed at The Taste and Smell Clinic, Washington, DC, between February 2001 and July 2005 and constitute studies on consecutive healthy subjects and patients. Studies were approved by the Institutional Review Board of the Georgetown University Medical Center.

Parotid saliva was collected from 61 healthy volunteers, aged 18 to 75 years (mean ± SEM, 50 ± 5 years). The volunteers were 40 men, aged 23 to 73 years (51 ± 7 years), and 21 women, aged 19 to 69 years (49 ± 4 years), who were well and healthy, without any acute or chronic disease, and

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not taking any medication. Smell and taste function in each subject was within normal limits. Parotid saliva was also collected from 253 patients, aged 9 to 83 years (55 ± 3 years), with taste and smell dysfunction. Patients were all those with taste and smell dysfunction who had loss of smell. These included 104 men, aged 9 to 83 years (56 ± 2 years), and 149 women, aged 12 to 79 years (49 ± 4 years).

Saliva was collected in the morning hours with subjects abstaining from eating or smoking at least 2 hours before collection. Saliva was collected by placing a modified Lashley cup over the Stensen duct and maximally stimulating flow by lingual placement of reconstituted lemon juice (Borden, Tarrytown, NY) at 10-second intervals, as previously described [14,15]. In patients, saliva was collected immediately after completion of sensory tasting. Saliva was collected continuously over an 8- to 12-minute period until approximately 8 mL was collected. Flow rate was calculated by obtaining mean fluid weight per time of collection. Saliva was stored at −20°C until assayed.

Cyclic AMP and cGMP were measured by a sensitive spectrophotometric 96-plate enzyme-linked immunosorbent assay technique using kits supplied by R&D Systems (Minneapolis, MN). Mean variation of kit standards was less than or equal to 5% [16,17]. Protein was measured by obtaining spectrophotometric absorbance at 215 to 225 nm with the use of the extinction coefficient by a method previously described [14]; in this manner, protein in very small samples was estimated. Cyclic AMP and cGMP in picomole/concentration were expressed in 3 ways: per milliliter saliva, per milligram protein, and per milliliter flow rate.

To determine methodological reliability, cAMP and cGMP were determined in several ways. Duplicates of 6 saliva samples were determined on 20 occasions; the standard deviation of these samples varied from 0.007 to 0.038 for cAMP and 0.007 to 0.038 for cGMP, respectively; mean coefficients of variation varied from 1% to 10% for each moiety. Cyclic AMP and cGMP from 1 subject were determined on 12 separate occasions over a period of 2 years. The standard deviation for these determinations for cAMP (picomoles per milliliter) was 0.29 with a mean coefficient of variation of 3%; for cAMP (picomoles per milligram protein), 0.13 with a mean coefficient of variation of 4%; for cAMP (picomoles per milliliter flow rate), 0.49 with a mean coefficient of variation of 5%; for cGMP (picomoles per milliliter), 0.02 with a coefficient of variation of 6%; for cGMP (picomoles per milligram protein), 0.007 with a coefficient of variation of 7%; and for cGMP (picomoles per milliliter flow rate), 0.05 with a coefficient of variation of 10%.

Smell loss was measured by psychophysical techniques by use of a forced-choice, 3-stimuli, stepwise staircase technique in a fixed controlled design [8,11-14]. Efficacy of these techniques and results therefrom were previously documented in a double-blind clinical trial [18]. Four odors were used: pyridine or “dead-fish” odor, nitrobenzene or bitter-almond odor, thiophene or petroleum-based odor, and amyl acetate or banana oil odor. Detection thresholds (DTs), recognition thresholds (RTs), and magnitude estimation (ME) for each odor were determined in this fixed controlled design as previously described [8,11-14,18].

Odors were presented in the order of thiophene, amyl acetate, nitrobenzene, and pyridine. Odors were stored in 60-mL, wide-mouth, screw-capped amber bottles with 12 mL of test solution in each bottle. For each test, each patient was seated at a right angle to the test administrator and was shielded from any visual contact with the test materials. For each test, the patient was required to sniff the headspace above the solution in each bottle in a sequence of 3 stimuli in succession in a fixed, mixed design [19]. Two of the stimuli were emollient (water or light mineral oil), and one was emollient with odorant. Each of the 3 solutions was opened and closed in sequence with the patients sniffing about 2 to 3 cm above the edge of the open bottle for 2 to 10 seconds. Each sequence of 3 stimuli required 20 to 60 seconds, with a rest period of 5 to 20 seconds between each set of 3 stimuli.

The patient was required to perform 3 tasks after the presentation of each set of 3 stimuli. First, the patient determined which stimulus with odorant was different from the 2 emollients without odorant (*to detect a difference* among the 3 stimuli); second, the patient described the odorant in the odorant containing stimulus in words, that is, *recognizing and describing the character* of the odorant; and third, the patient estimated the intensity of the identified odorant using a scale from 1 to 100 (an estimate of *magnitude intensity*). On this scale, 100 was described as the most intense odorant of the odorant type previously experienced under normal conditions within that odor category. Thus, 1 was the least intense, 100 was the greatest, and any intensity between 1 and 100 was judged accordingly.

Testing always began at what had been previously determined to be the upper limit of normal detection (10^{-5} mol/L for each odorant) [14,18]. If the patient detected and recognized the odorant correctly at this concentration, the odorant concentration was decreased stepwise (10^{-6}, 10^{-7}, 10^{-8}, and 10^{-9} mol/L) until the patient could no longer correctly detect or recognize any difference among the 3 stimuli. If the patient could neither detect nor recognize the odorant correctly at 10^{-5} mol/L, the odorant concentration was increased stepwise (10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, and 10^{0} mol/L and absolute) until the patient could detect and recognize the odorant correctly. Using this staircase, turn-around technique, odorant concentrations were either increased or decreased until the patient detected and recognized correctly the odorant as different from emollient 2 out of 3 times at 1 concentration and could not do so at the next lower or higher concentration. Thus, if the patient could neither detect nor recognize correctly 10^{-5}, 10^{-4}, and 10^{-3} mol/L odorant but could both detect and recognize correctly odorant at 10^{-2} mol/L, the next stimulus presented was the next lower concentration, 10^{-3} mol/L. If the patient could once again neither detect nor recognize correctly the stimulus

Table 1
Classification of severity of odor loss

Severity of smell loss	DT	RT	ME
Anosmia	0	0	0*
Hyposmia			
Type I	−	0	0*
Type II	−	−*	≥ 0 < normal
Type III	+	+*	≥ 0 < normal

Detection threshold [9-11,14,18], RT [9-11,14,18], and ME [14,18]. 0 indicates inability to both detect and recognize correctly any odorant at any concentration; 0*, the patient is unable to recognize correctly any odorant at any concentration, rendering any intensity measurement invalid or 0; DT −, inability to detect any odorant at $\leq 10^{-5}$ mol/L (responses are $\geq 10^{-4}$ mol/L for all odorants); RT −*, inability to recognize correctly any odorant at $\leq 10^{-2}$ mol/L (responses are $\geq 10^{-1}$ mol/L for all odorants); DT +, ability to detect all odorants at $\leq 10^{-5}$ mol/L; RT +*, ability to recognize correctly all odorants at $\leq 10^{-2}$ mol/L. Magnitude estimation, normal (pyridine, <61%; nitrobenzene, <46%; thiophene, <63%; amyl acetate, <48%) [10,14,18].

at 10^{-3} mol/L, the stimulus at 10^{-2} mol/L was once again presented. If the patient once again detected and correctly recognized the stimulus at 10^{-2} mol/L, this part of the test was completed because the patient gave 2 incorrect responses at 1 stimulus concentration and 2 correct responses at the next higher concentration. The odorant concentration at 10^{-2} mol/L was considered the DT and RT.

Magnitude estimation determination began in healthy subjects at what had previously been determined to be the lower limit of normal odor recognition (10^{-2} mol/L), although measurements of odorant stimulus magnitude was always requested along with each statement of odorant detection and recognition. Thus, the actual ME determination was calculated using the intensity and correct recognition response by averaging intensity given from odorant concentrations 10^{-2}, 10^{-1}, or 10^{0} mol/L and absolute. The mean of these numbers comprised the ME response.

Because interval presentations for DT and RT were not equal, odor concentrations were transformed to a linear scale (eg, 10^{-9} mol/L = 1, 10^{-8} mol/L = 2, … absolute = 11) so that a scale of equal units and an absolute zero was obtained [14,18,19]. Using this scale, DT and RT for each patient were assigned a number, and mean ± SEM of responses for each patient group was calculated. Mean ± SEM for ME for each patient group was similarly determined (in percentage) using the individual values previously determined.

Based upon results of DT, RT, and ME, smell loss degree was classified as shown in Table 1. This classification indicates that patients with anosmia have the greatest severity of smell loss; those with type I hyposmia, the next greatest; those with type II hyposmia, the next greatest; and those with type III hyposmia, the least severe loss. Related to severity of smell loss, acuity decreased from greatest to least degree of smell loss such that anosmia > type I hyposmia > type II hyposmia > type III hyposmia (Table 1).

Each measurement of saliva cAMP and cGMP was placed into 1 of the 4 categories of smell loss severity. Afterward, mean ± SEM for cyclic nucleotides in each group of loss severity was calculated. Differences between group means were calculated using Student *t* tests. Biochemical determination of each salivary cAMP and cGMP level was also correlated with each of the 4 smell loss categories by use of a Spearman rank correlation technique, and significance of correlation was determined.

All studies of saliva cyclic nucleotides were initially coded. Results of smell loss classification were obtained independent of any saliva cyclic nucleotide results. Only after all patient classifications of smell loss were defined were saliva studies uncoded and correlated.

3. Results

Levels of salivary cAMP in all patients with smell loss were significantly lower than those in healthy subjects (Table 2) [8]. When categorized by loss severity, there was a consistent decrease in salivary cAMP and cGMP with increased loss severity (Table 2).

Although only 2 patients with anosmia were studied, mean levels of both cAMP and cGMP were lower than normal and lower than in any other smell loss type (Table 2).

Table 2
Cyclic AMP and cGMP in parotid saliva in patients with taste and smell loss classified by severity of loss

Smell loss	cAMP			cGMP			Protein	Flow rate	Age
	pmol/mL	pmol/mg protein	pmol/min flow rate	pmol/mL	pmol/mg protein	pmol/min flow rate	mg/dL	mL/min	y
Anosmia (2)	0.22	0.06	3.70	0.62	0.01	0.06	0.62	3.70	56
Hyposmia									
Type I (54)	0.78 ± 0.09[a]*	0.26 ± 0.02*	3.01 ± 0.09	0.76 ± 0.09‡	0.047 ± 0.010†,‖	0.18 ± 0.04	0.76 ± 0.09‡	3.01 ± 0.09	47 ± 2
II (189)	0.87 ± 0.04*	0.28 ± 0.02†	3.12 ± 0.02	0.68 ± 0.07§	0.049 ± 0.005*,‖	0.22 ± 0.05	0.68 ± 0.07§	3.12 ± 0.02	57 ± 1
III (8)	1.01 ± 0.12*	0.33 ± 0.06†	3.09 ± 0.14	0.62 ± 0.15	0.091 ± 0.009	0.45 ± 0.09	0.62 ± 0.15	3.09 ± 0.14	51 ± 5
Healthy subjects (61)	2.00 ± 0.19	0.63 ± 0.06	3.17 ± 0.18	0.92 ± 0.05	0.066 ± 0.009	0.23 ± 0.04	0.92 ± 0.05	3.17 ± 0.18	50 ± 5

Numbers in parentheses indicate number of subjects.
With respect to healthy subjects: *$P < .001$, †$P < .005$, ‡$P < .02$, and §$P < .01$.
With respect to type III hyposmia: ‖$P < .005$.
[a] Mean ± SEM.

Table 3
Cyclic AMP and cGMP concentrations in parotid saliva of men and women with anosmia and hyposmia

	cAMP			cGMP			Protein	Flow rate	Age
	pmol/mL	pmol/mg protein	pmol/min flow rate	pmol/mL	pmol/mg protein	pmol/min flow rate	mg/dL	mL/min	y
Men									
Anosmia (2)	0.22	0.06	0.35	0.05	0.01	0.06	3.70	0.62	56
Hyposmia									
Type I (28)	0.75 ± 0.11[a]	0.24 ± 0.04	1.03 ± 0.15	0.13 ± 0.03	0.03 ± 0.012	0.18 ± 0.06	3.05 ± 0.09	0.74 ± 0.05	50 ± 3[§]
Type II (109)	0.84 ± 0.07	0.27 ± 0.03	1.25 ± 0.14	0.20 ± 0.04	0.64 ± 0.023	0.30 ± 0.07	3.14 ± 0.09	0.67 ± 0.03	58 ± 2
Type III (2)	0.99	0.36	1.10	0.39	0.14	0.43	2.78	0.90	61
Women									
Hyposmia									
Type I (26)	0.79 ± 0.09[‡]	0.27 ± 0.03	1.07 ± 0.10*	0.16 ± 0.03	0.054 ± 0.010	0.22 ± 0.05	2.97 ± 0.11[‖]	0.74 ± 0.05[‡]	46 ± 3[†]
Type II (80)	0.97 ± 0.05	0.30 ± 0.02	1.62 ± 0.09[¶]	0.15 ± 0.01	0.047 ± 0.004	0.25 ± 0.02	3.25 ± 0.05[#]	0.60 ± 0.02	56 ± 1
Type III (6)	1.07 ± 0.16	0.42 ± .015	1.98 ± 0.07	0.21 ± 0.10	0.083 ± 0.040	0.38 ± 0.09	2.54 ± 0.10	0.54 ± 0.10	50 ± 16

Numbers in parentheses indicate number of subjects.
With respect to type II hyposmia: *$P < .001$, [†]$P < .005$, [‡]$P < .02$, [§]$P < .05$, and [‖]$P < .01$.
With respect to type III hyposmia: [¶]$P < .02$ and [#]$P < .05$.
[a] Mean ± SEM.

Patients with the next most severe type of hyposmia (type I) had significantly lower salivary levels of cAMP than did both patients with the next least severe smell loss type (type II) and healthy subjects. When categorized by flow rate, cAMP was significantly lower than in patients with types II and III hyposmia. When categorized with respect to protein, cAMP in patients with type II hyposmia, although not significantly different from that in patients with type III hyposmia, was 6% lower (Table 2) and, when characterized by flow rate levels, was 7% lower (Table 2).

Levels of cGMP were 2 to 3 times lower than in healthy subjects (Table 2). Although levels were not significantly different, cGMP in patients with type I hyposmia were one half that in patients with type III hyposmia. In patients with type II hyposmia, levels were 46% those with type III hyposmia. Mean salivary cGMP for patients with type III hyposmia (the least severe type of hyposmia), although 27% lower than levels of healthy subjects, were not significantly different from normal levels (Table 2).

Salivary levels of cAMP were higher than those for cGMP in all patient groups except for the 2 patients with anosmia in whom measurements of both cyclic nucleotides were close to zero. However, levels of cAMP in the patients were only 3 to 5 times higher than cGMP, whereas in healthy subjects, this difference ranged from 7 to 10 times higher.

Correlation of salivary cAMP with smell loss type was $r_s = -0.83$ ($P < .001$); correlation of salivary cGMP with smell loss type was $r_s = -0.79$ ($P < .001$). These demonstrate that the higher the level of salivary cGMP or cGMP, the less severe the loss of smell acuity among these patients.

Saliva flow rate was significantly lower than normal only in patients with type II hyposmia.

Salivary cyclic nucleotides were also determined in patients with smell loss classified by both degree of smell loss and sex (Table 3). There were no significant sex differences in either salivary cAMP or cGMP related to degree of smell loss. However, both men and women with type II hyposmia were significantly older than men and women with type I hyposmia; salivary protein was significantly lower in women with type I hyposmia than in those with type II hyposmia (Table 3).

4. Discussion

Although we have previously reported lower than normal levels of salivary cAMP and cGMP in patients with taste and smell dysfunction [8], we have not previously related these decreased levels directly to clinical parameters that were determined independently from these biochemical studies.

Several clinical findings result from these studies. First, severity of smell loss was negatively correlated with salivary levels of cAMP and cGMP. Second, salivary levels of cAMP were slightly more negatively correlated with smell loss degree than were levels of cGMP. Third, results indicate that quantitative psychophysical measurements of smell loss can be correlated with measured levels of salivary cyclic nucleotides. This is the first demonstration of specific biochemical changes in saliva correlated with quantitative measurements of olfactory acuity and reflects an important method by which to identify patients with smell loss.

These results reflect an independent technique by which the classification of smell loss severity we previously reported [10,11] can be supported. This classification of smell loss was first proposed in 1965, determined by anatomical and surgical studies in which the olfactory epithelium was either surgically excised [9,20-22] or inhibited secondary to anatomical denervation [23,24]. We have since expanded this classification to include all patients with smell loss; and indeed, all patients with these losses fall

within this classification. This classification was found to be useful not only to describe degree of smell loss but also to predict response to therapy because treatment of patients with type II hyposmia, a lesser degree of hyposmia than type I hyposmia, was generally more effective than treatment of patients with type I hyposmia, the more severe degree of hyposmia [25].

Both cAMP and cGMP have been shown to play roles in both sensory and neural function. Cyclic AMP acts as a growth factor in cultured nerve cells inducing neurite outgrowth [26,27] similar to that of nerve growth factor in cultured chick ganglion cells [28]. In lesioned zebra fish, cAMP induced regeneration of neuronal activity and normal axonal function [29,30]. Cyclic AMP phosphodiesterase (PDE) activity [31] and nerve growth factor activity [32,33] have been demonstrated in purified taste bud membranes and have been shown to be influenced by hormones [34] and divalent metal ions, including zinc [35]. Odorants activate olfactory receptor neuron transduction via a cAMP signaling cascade that results in opening of nonselective cyclic nucleotide-gated channels involved with subsequent Ca^{2+} influx through cyclic nucleotide-gated Cl^{-} activated channels that amplify the transduction signal [36]. Activation of these cyclic nucleotide-gated channels by odorants is highly cooperative [37] and is present in many species [38]. Local PDE activity has been demonstrated to regulate cAMP activation of olfactory-stimulated neuronal activity [39].

Adenylyl cyclases have been found in both olfactory epithelium [4] and taste buds [4]. Cyclic nucleotides have been previously found in nasal mucus [12,40,41] and have been correlated with smell loss severity in studies similar to those reported here [13]. Decreased cAMP in parotid saliva in the present patients is consistent with decreased neural growth and development in the olfactory system [1,12,40] and presumably in the taste system as well. This decreased growth may be manifested by the pathologic apoptotic changes previously observed in taste bud [42] and in olfactory epithelia [11] in patients with taste and smell loss. We have used PDE inhibitors to treat patients with taste and smell dysfunction and low levels of salivary cAMP and cGMP. One such drug, theophylline, whose administration resulted in changes in saliva cAMP [43], restored taste and smell function in some patients as measured by psychophysical methods [11,14,44] and by tests of smell function using brain functional magnetic resonance imaging [45]. Theophylline has been shown to exhibit an antiapoptotic effect in patients with taste and smell dysfunction in whom smell function has been restored [46]. An effect similar to that observed with theophylline has also been observed through use of a more specific PDE inhibitor, cilostazol (RI Henkin and I Velicu, unpublished observations). Underlying mechanisms for this effect are through the putative action of PDE inhibitors in stimulating growth and maturation of stem cells in the taste and smell systems as suggested by use of other therapeutic agents [47] and by inhibition of apoptotic properties of cells in these systems [46].

Cyclic GMP has also been shown to play a specific role in taste [1,4] and smell [5-8] function. It plays multiple roles in these [5-8] and in other [48-50] sensory systems. It is part of an enzymatic pathway in visual signal transduction [49,51], and PDE inhibitors act in a similar manner to cGMP itself [51,52].Cyclic GMP–dependent protein kinase has been shown to regulate proliferation of sensory neurons [52]. Although cAMP may be the primary second messenger mediating olfactory signal transduction in mammals [5-8], cGMP plays other roles in olfactory signal transduction including adaptation, neuronal development, and long-term cellular responses [49-52]. Cyclic GMP PDE activity is decreased by odorant stimulation [53] and is sensitive to cGMP concentration [54]. Odorant-activated cGMP uses several pathways in olfactory sensory neurons [55], expressing specific types of messenger RNA [56] and increasing spike frequency in response to strong odorant stimuli [57].

We measured an oral fluid, saliva, yet related measurements of salivary cyclic nucleotides to smell loss. At first glance, this connection may appear incongruous. However, most patients who complain of loss of taste do so not so much because they cannot taste the basic qualities of salty, sweet, sour, or bitter but because they cannot obtain flavor from food, which relates more to smell loss [11]. In addition, our studies of biochemical composition of saliva and nasal mucus indicate that many of the chemical moieties present in saliva are also present in nasal mucus, albeit concentrations may differ considerably [58].

The present results describe both an independent biochemical foundation for a classification of smell loss and a systematic biochemical basis for defining clinical smell loss. Because there are as many as 20 million patients in the United States who experience chronic loss of smell [59] from multiple causes [11,14,60], establishment of this relationship offers an important biochemical and clinical formulation by which a large number of these patients can be identified and effective treatment can be considered [44,45,61]. These studies are in some respects similar to the initial seminal studies of biochemical typing of blood lipid abnormalities [62,63] to assess risk classification of patients with cardiovascular disease that hastened both understanding of its pathology and its treatment.

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