Published in final edited form as:

Arch Oral Biol. 2010 March ; 55(3): 249–254. doi:10.1016/j.archoralbio.2009.12.007.

The Effect of Sucrose on Plaque and Saliva Urease Levels *in vivo*

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Abstract

Objective—Dietary sugar exposures induce an immediate drop of the plaque pH. Based on *in vitro* observations, it was hypothesized that oral bacteria may rapidly respond to this environmental change by increasing the activity or expression of alkali-generating pathways, such as the urease pathway. The objective of this exploratory *in vivo* study was to determine the short-term effect of a brief sucrose exposure on plaque and saliva urease activity and expression, and to relate this effect to caries experience.

Methods—Urease activity levels were measured in plaque and saliva samples collected from 20 children during fasting conditions and 30 minutes after rinsing with a sucrose solution. *Streptococcus salivarius ureC*-specific mRNA in saliva was quantified using Real-Time RT-PCR. The impact of host-related factors, such as age, gender, sugar consumption, salivary mutans streptococci levels and caries status on urease activity was evaluated.

Results—Plaque urease activity under fasting conditions was higher in subjects with low caries and mutans streptococci levels. This difference was not observed after the sucrose exposure. The response of urease to sucrose *in vivo* did not depend on caries experience or salivary mutans levels. Significant increase in urease activity of plaque and saliva after exposure to sucrose was observed only in the subjects who had low urease levels at baseline.

Conclusions—The findings of this exploratory study suggest that plaque urease activity may have an important long-term influence in caries development but not during a cariogenic challenge.

Keywords

Urease; genetic responses of oral bacteria; caries; translational

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Introduction

Despite the many efforts to improve oral health, dental caries remains a significant health problem for children and adults worldwide. To improve the assessment of caries risk and treatment planning for each individual, a better understanding of the biological processes leading to the onset of caries is warranted. It has been well documented that the development of caries requires a decrease in dental plaque pH to acidic values, which occurs as a result of the glycolytic metabolism of dietary carbohydrates by oral bacteria (24). The intensity and duration of plaque acidification is therefore an important risk factor for caries and it differs among caries-free and caries-active individuals (24). This difference may be due, among other things, to a higher concentration of ammonia in oral biofilms of caries-free compared to caries-active individuals (4,14). One important source of ammonia in the mouth is the action of bacterial ureases (4), which hydrolyze urea present in plaque and saliva into ammonia and carbon dioxide, causing a significant pH rise (4,6,7,11,12,15,23). Recently, a significant inverse association between plaque urease activity and caries experience has been demonstrated in children and adults (9,13,17), suggesting that a reduced ability to generate ammonia from urea in dental plaque may be an important caries risk factor.

In vitro studies have shown that urease expression in certain oral bacteria is not constitutive, but can be significantly increased under caries-promoting environmental conditions. For example, in *Streptococcus salivarius* urease expression can be increased up to 300-fold in acidic pH and during excess of carbohydrate (5,6,21). Urease expression in *Actinomyces naeslundii* can increase up to 50-fold during growth under nitrogen-limitation, a condition that oral bacteria may experience when carbohydrates are present in excess (16). Based on these *in vitro* observations, ongoing clinical studies at the University of Puerto Rico are examining the long-term effects of sugar consumption on urease activity levels *in vivo*, as it relates to caries development in children (3). Dietary sugar exposures are known to induce an immediate drop on the pH of the dental plaque, and based on the available knowledge, it was hypothesized that oral bacteria may rapidly respond to this environmental change by increasing the activity or expression of alkali-generating pathways, such as the urease pathway. Furthermore, it was hypothesized that this response may be associated with the overall caries experience of a child. To test this hypothesis, the urease activity in plaque and in saliva of children was measured during fasting conditions, and thirty minutes after a brief sucrose exposure. In addition, the expression of the *ureC* gene of *S. salivarius*, which encodes the large structural subunit of urease, was quantified in saliva, as this gene has been shown to be highly responsive to pH and carbohydrate availability *in vitro* (6). The impact of host-related factors, such as age, gender, sugar consumption, salivary mutans levels and caries experience on urease activity under fasting conditions and after exposure to sucrose was evaluated. To our knowledge, this is a first study looking at the genetic responses of oral bacteria to environmental stimulation at the clinical level.

Methods

A total of 20 healthy children ages 5 to 12 years were recruited for this study. The sample size was determined empirically based on related ongoing studies at the University of Puerto Rico (UPR). A child was not eligible if he/she (i) had received antibiotics within the last three months, (ii) was using any medications at the time of the study, or (iii) was wearing orthodontic appliances. Children who were able to read and write assented to their participation to the study. Parents of all the participants authorized the voluntarily participation of their children by signing a consent form approved by the Institutional Review Board of the UPR Medical Sciences Campus.

All samples were collected in the morning between 8 and 11 am. Subjects were required to refrain from eating and drinking anything but water, and to refrain from oral hygiene procedures for a minimum of 8 hours before the study (usually since the night before). A baseline (pre-rinse) sample of whole, unstimulated saliva (5 mls) was initially collected using a mucous trap attached to the dental suction (9). Supragingival plaque was then collected from all available smooth dental surfaces on the upper and lower right quadrants and pooled into a micro-centrifuge tube. Following the collection of the baseline plaque and saliva samples, subjects were asked to rinse their mouth for one minute with 5 ml of a sterile 10% sucrose solution (Fisher Scientific, Fair Lawn, NJ) in dH₂O. Post-rinse unstimulated saliva and plaque samples were collected 30 minutes later with the same method, except that plaque samples were now collected from the quadrants on the opposite side of the mouth (upper left and lower left). After the collection of the post-rinse samples, subjects were asked to brush their teeth with fluoride-containing toothpaste. A dental exam was finally performed as described below.

Plaque and saliva samples were kept on ice during collection and then immediately transferred to the laboratory. Saliva samples were divided into 1 ml aliquots. Plaque samples were resuspended in 300 µl of 10 mM sodium phosphate buffer and aliquoted. All samples were then snap-frozen using a dry ice/ethanol mix and stored at -70°C within 15 minutes from collection.

Urease activity

Urease activity was measured as previously described (13,17). Each plaque and saliva sample was assayed in identical triplicate reactions. A control reaction without urea was included for each sample in order to determine the background levels of ammonia present from sources other than urease activity. Urease activity was normalized to protein content as determined by the method of Bradford (2) (Sigma, St. Louis, MO). The units used to measure urease activity were defined as µmoles of urea hydrolyzed min⁻¹ (mg of protein).

Dental Caries

Dental caries were detected with the Fiber-Optic Trans-Illumination (FOTI) method (SCHOTT North America Inc., Southbridge, MA) (19) using Ekstrand's criteria (8). A dmfs (DMFS) index was then calculated in order to express the overall caries experience of each child. A previously calibrated examiner performed all dental examinations.

Levels of mutans streptococci in saliva

Levels of mutans streptococci in saliva were determined by plating on duplicate Mitis Salivarius agar plates (Beckson-Dickinson, Sparks, MD) supplemented with 20% sucrose and bacitracin (0.2 units/ml) (10) (MSB). The number of mutans streptococci were expressed as CFUs per ml of saliva and categorized from 0 to 4 as follows: 0: 0 cfu; 1: ≤10⁴ cfu/ml; 2: ≤10⁵ cfu/ml; 3: ≤10⁶ cfu/ml; and 4: >10⁶ cfu/ml.

Sugar Consumption

Sugar consumption was measured using a 24-hour dietary recall completed by the parents. Using the information from this record, each sugar-containing food consumed by the child was classified into one of three categories: "liquid", "solid & sticky", and "slowly dissolving". A final score was calculated for each child multiplying the sum of food exposures in each category by a factor specific for the category and adding up the scores (18).

RNA isolation

RNA was isolated from saliva samples using the FastRNA® Pro Blue Kit (MP Biomedical, Solon, OH, USA) according to supplier's instructions with minor modifications. Saliva

samples (2 ml) were transferred to the matrix tube (FastPrep® Instrument) and homogenized in a Bead Beater using two cycles of 20 seconds, with cooling on ice during the interval. The samples were incubated at 65°C for 20 minutes and then centrifuged for 5 minutes at 14,000 g in a refrigerated micro centrifuge (Labnet International, Woodbridge, USA). The upper phase was transferred to a new tube containing 300 µL of chloroform and vortexed for 10 seconds. After new centrifugation for 15 min at 14,000 g at 4°C, the aqueous phase was transferred to a new tube containing 500 µL of isopropanol and incubated at -20°C for 60 minutes. After centrifugation for 15 min at 14,000 g at 4°C, the pellet was washed twice with 500 µL of 75% ethanol (made with DEPC-H₂O). The pellet was then air-dried and resuspended in 25 µL of DEPC-H₂O. RNA concentration was determined spectrophotometrically in triplicates, and 1 µg of RNA was run in a formaldehyde gel to verify RNA quality.

Real-time quantitative PCR

Oligonucleotide primers used in this study were designed using DNA mfold (<http://www.bioinfo.rpi.edu/applications/mfold/old/dna/>) and Beacon Designer 2.0 (Premier Biosoft International, Palo Alto, CA), as described elsewhere (1,17). Species-specific primers were designed from the *ureC* gene of *S. salivarius* (F-AGG TTC AGG TGG TGG ACA TGC; R-TTG TGG TGT ATG GGT TGA TTG GG; PCR product = 98 bp), and the universal oligonucleotide primers for a broad range of bacterial 16S rRNAs were also utilized (17,20) (F-ACT ACG TGC CAG CAG CC, R-GGA CTA CCA GGG TAT CTA ATC C; PCR product = 296-300 bp). To obtain cDNA, 1 µg of three independent RNA samples and the iScript kit containing species-specific primers (Bio-Rad, Hercules, CA) were used. Reverse transcription and real-time reverse transcriptase PCR (RT-PCR) was carried out according to protocols described elsewhere (1). Standard curves for each gene were prepared as described by Yin *et al* (26) and used in every Real-time quantitative RT-PCR run. A range of 10¹-10⁸ copies was found to be adequate for all the genes examined. Real-time PCRs were carried out in an iCycler iQ real-time PCR detection system (Bio-Rad Laboratories, Inc., Hercules, CA) using iQSYBR green supermix (BioRad Laboratories, Hercules, CA). *S. salivarius ureC* copies were expressed as % of total 16S rRNA copies.

Statistical analysis

Descriptive statistics including means, standard deviation, and median values were computed in order to have an epidemiological profile of the sample. Logarithmic transformations were used to normalize plaque urease. *UreC* mRNA levels were normalized to 16S rRNA levels. The proportions of *ureC* to total RNA were normalized using the logit transformation ($\log(p/1-p)$). The one-sided unpaired t-test for independent samples was used to compare the baseline values of plaque urease, saliva urease and *S. salivarius ureC* copies by gender, age, sugar consumption, caries scores, and salivary mutans levels. To make this comparison, age, sugar consumption, caries scores and salivary mutans levels were dichotomized by the median. Similar procedure was performed for post-rinse values of plaque urease, saliva urease and *S. salivarius ureC* copies. The Spearman rho coefficient was used to evaluate the correlations between plaque urease, saliva urease and *S. salivarius ureC* copies before and after the sucrose exposure. Finally, the one-sided paired t-test was used to compare pre- and post- rinse urease levels (plaque and saliva) and *S. salivarius ureC* copies stratified by gender, age (above and below the median), caries levels (above and below the median), sugar score (above and below the median), salivary mutans levels (above and below the median) and baseline urease levels (above and below the median). Data were analyzed using STATA version 10 program (StataCorp LP, College Station, TX).

Results

Table 1 presents the descriptive statistics of all study variables. There was a balanced distribution of gender, age, sugar scores and salivary mutans levels in the study group. Caries scores did not have a normal distribution, however 45% of the children were caries-free ($d_4mfs+D_4MFS=0$), while the other 55% exhibited a wide range of caries scores (1 to 26 with median $d_4mfs+D_4MFS=1$).

Table 2 presents plaque and saliva urease activity levels and *S. salivarius ureC* mRNA levels in the 20 subjects at baseline (fasting conditions) and after the sucrose rinse. The data are shown for the group as a whole, as well as stratified by baseline plaque urease, baseline saliva urease, caries levels, and salivary mutans levels. The subgroups were defined by dichotomizing these variables by the median values. Initially, the mean baseline values of plaque urease, saliva urease and *S. salivarius ureC* were compared in the different subgroups. The same comparisons were repeated for mean post-rinse values of the same variables. Baseline values of plaque urease, saliva urease and *S. salivarius ureC* copies were compared against their post-rinse values using paired t-test. These comparisons were performed in the group as a whole, and within the subgroups. The data of the analysis by gender, age and sugar consumption are not included in Table 2 because no significant differences were observed among the corresponding subgroups (un-paired t-test and paired t-test $P>0.05$).

Urease activity under fasting conditions (baseline) was significantly lower (t-test: $P=0.015$) in the plaque of children with caries levels above the median ($D_4MFS>1$), and in those with high levels of mutans streptococci in their saliva ($\geq 10^5$ CFU/ml, t-test: $P=0.04$) compared to children with low caries ($DMFS\leq 1$) and to those with low salivary mutans levels ($<10^5$ CFU/ml). Urease activity in saliva under fasting conditions did not differ by levels of mutans streptococci or caries levels. *S. salivarius ureC* levels were significantly higher in children with high saliva urease activity, compared to those with low urease activity in their saliva ($P=0.003$). Furthermore, *S. salivarius ureC* expression correlated significantly with salivary urease activity levels both under fasting conditions (Spearman $\rho=0.78$, $P=0.007$) and after exposure to sugar (Spearman $\rho=0.71$, $P=0.02$). Similar to plaque urease activity, *S. salivarius ureC* levels at baseline were much higher (but not statistically) in subjects with low caries and mutans streptococci levels (Table 2).

In the post-rinse samples, no significant differences in plaque urease, saliva urease or *S. salivarius ureC* expression were observed by age (data not shown), gender (data not shown), sugar consumption (data not shown), caries levels, or salivary mutans levels. A significant increase in plaque urease activity was observed only in subjects who had low plaque urease activity levels (below the median) at baseline (paired t-test: $P=0.037$). Similarly, saliva urease activity increased significantly after sugar challenge only in subjects who had low salivary urease levels (below the median) at baseline (paired t-test: $P=0.018$). Expression of *ureC* in *S. salivarius* showed a tendency to increase after the sucrose exposure in all subgroups. However, this increase was not statistically significant (paired t-test: $P>0.5$).

Discussion

The generation of ammonia from urease activity by oral bacteria increases dental plaque pH, and therefore, it is believed to inhibit the onset of dental caries. The best so far characterized urease enzymes in oral bacteria are the ones produced by *S. salivarius* and by *Actinomyces naeslundii*. The expression of ureases in these oral bacteria can be increased 50 to 300-fold under increased carbohydrate concentrations or acidic pH, conditions that are clinically analogous to a cariogenic challenge (5,6,16,21). To determine if urease activity in plaque and/or in saliva is induced during a cariogenic exposure *in vivo*, we measured urease activity and

S. salivarius ureC mRNA during fasting conditions and after a short rinse with a sucrose solution, which is known to induce an immediate and reversible drop in plaque pH (24). In contrast to the *in vitro* experiments, no significant increase in urease activity of plaque or saliva was observed in this *in vivo* study after the sucrose rinse when all subjects were considered. However, plaque urease activity increased significantly in the subjects that had low levels of plaque urease (below the median) under fasting conditions. Similarly, urease activity in saliva increased significantly in the subjects that had low salivary urease levels (below the median) at baseline. The expression levels of *S. salivarius ureC* mRNA levels in saliva showed a clearer trend for induction following exposure to sugar. This increase was not statistically significant likely due to the small sample size.

There are a number of possible explanations for the differences between the *in vitro* data and the observations in this *in vivo* experiment. First, *in vitro* studies have been conducted on single species cultures of *S. salivarius* and *A. naeslundii* (5,6,16,21). In contrast, the natural plaque and saliva samples used here represent multi-species bacterial populations, which may exhibit high variability with respect to the types and proportions of ureolytic species they contain. The expression of *ureC* in *S. salivarius* showed a more consistent trend towards induction in response to sucrose, which is in accordance with *in vitro* observations in the same organism. *S. salivarius ureC* mRNA levels in saliva correlated well with salivary urease activity levels, both in the fasting samples and after the sucrose exposure. This suggests that *S. salivarius* may be the predominant ureolytic organism in saliva. In contrast, dental plaque may contain a variety of unidentified ureolytic species, as previously suggested (22), in which urease expression may not be regulated by pH or carbohydrate availability. In fact, most known bacterial ureases in nature are regulated either by nitrogen shortage or substrate availability (15).

Another possible explanation for the fact that no significant increase in urease levels was observed in this *in vivo* study may be that the growth rates of bacteria in mature natural dental plaque are much slower when compared to the exponentially growing cells used in *in vitro* experiments, (25). Even though gene transcription is not dependent on cell division, one can argue that cells with slow metabolism may not respond to environmental challenges with the same speed as rapidly growing cells. In fact, it has been observed that induction of urease expression by low pH in *S. salivarius* in a continuous culture system is much more significant in rapidly growing cells, compared to cells growing at slower dilution rates (5). It is also possible that the time of sugar exposure and consequent pH drop used in this study was not sufficient to achieve maximal induction of the urease genes in the oral samples, although high increases were observed in particular samples, as explained below. Most importantly, in contrast to the well-defined conditions of *in vitro* experiments, *in vivo* studies can be confounded by a variety of unknown host factors. To begin evaluating how host-related factors may influence the expression of urease in response to sugar, we studied the effects of gender, age, levels of mutans streptococci in saliva, sugar consumption and caries levels.

Low levels of mutans streptococci in saliva and low caries scores were associated with higher urease activity in fasting plaque. These observations are in agreement with data from ongoing (3) and recently published studies (13,17) providing further support to the hypothesis that a reduced ability to produce alkali from urea in plaque may be associated with increased caries susceptibility. Interestingly, these differences were observed only in the baseline (fasting) data, and not in the post-sucrose values. In addition, there were no significant differences among subjects with low caries and mutans levels vs. high caries and high mutans levels in terms of their urease response to the sugar challenge. In other words, children with low caries and mutans levels did not have a more significant induction in urease activity during exposure to sucrose compared to children with high caries and mutans levels. These observations suggest that the importance of urease in caries development is probably related to the long-term effects of ammonia on the plaque pH and in oral ecology.

Statistically significant increases in plaque and saliva urease levels were observed only in the subjects who had low urease levels at baseline. A possible explanation for this observation is that in subjects with high urease activity at baseline urease is already fully induced. It is also possible that subjects with low urease activity may experience a more pronounced acidification in their plaque and saliva following the sugar rinse and, consequently, a greater increase in urease expression compared to subjects who have more urease activity at baseline. In order to confirm this observation it would be necessary to measure the pH before and after the sucrose rinse, which was not done in this study. It would also be important to include a control group rinsing with water alone, although this could introduce additional variability in the design. Alternatively, the lower baseline levels in these subjects could arise from the presence of host factors that suppress urease gene expression and/or that inhibit the urease enzyme.

Designing and carrying out clinical studies to test hypotheses that have originated from *in vitro* observations can be complex and can have many limitations pertaining to subject recruitment and compliance, controlling for potentially confounding host factors, and increased variability. However, these types of translational studies are important because they provide the opportunity to identify such factors and to understand more thoroughly the biological processes involved in human diseases. To our knowledge, this is the first study to look at the genetic responses of oral bacteria to environmental stimulation directly at the clinical level. Despite the limitations, the findings of this exploratory study provide further insight in the clinical role of a potentially important caries risk factor. Emerging data from ongoing prospective clinical studies in the UPR School of Dental Medicine will help elucidate the role of this new risk factor in oral ecology and in caries development.

Acknowledgments

The authors would like to thank Dr. Ronald Billings, University of Rochester for the critical review of the manuscript. The study described was partially supported by K23 DE015285 and by RO1 DE10362 from the National Institute for Dental and Craniofacial Research, by RCMI grant #G12 RR 03051, by RCRII IP20 RR 11126, and by R25 RR 017589, which is co-funded by National Center for Research Resources, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Center on Minority Health and Health Disparities, National Heart, Lung, and Blood Institute, National Institute on Aging, National Institute of Diabetes and Digestive and Kidney Diseases, National Institute on Drug Abuse, and National Institute of Child Health and Human Development of the National Institutes of Health." "Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

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Table 1
Distribution of Study Variables (n=20)

Variable	Mean±SD	Median	Min-Max
Gender	NA	NA	NA
Males: 8 (40%)			
Females: 12 (60%)			
Age (years)	8.8±2.3	9	5-12
Caries Score (d ₄ mfs+D ₄ MFS)	4.2±7.1	1	0-26
Sugar Score	14.6±4.4	15	6-24
Salivary Mutans	2.3±1.3	3	0-4
Plaque Urease Fasting (μmoles urea/min/mg)	2.72±2.42	1.74	0.47-9.25
Plaque Urease Post-rinse (μmoles urea/min/mg)	4.05±7.28	2.3	0.47-34.15
Saliva Urease Fasting (μmoles urea/min/mg)	0.47±0.35	0.34	0-1.24
Saliva Urease Post-rinse (μmoles urea/min/mg)	0.5±0.3	0.45	0.06-1.08
<i>S. salivarius ureC</i> Fasting (% 16S rRNA copies)	0.37±1.04	0.02	0-3.5
<i>S. salivarius ureC</i> Post-rinse (% 16S rRNA copies)	0.8±1.35	0.02	0.001-3.34

Table 2
Comparisons of Plaque Urease, Saliva Urease, and *S. salivarius ureC* in Baseline and Post-rinse Samples

Groups	Plaque Urease (n=20)		Saliva Urease (n=20)		<i>S. salivarius ureC</i> (n=11)	
	Baseline (mean±SE)	Post-rinse (mean±SE)	Baseline (mean±SE)	Post-rinse (mean±SE)	Baseline (mean±SE)	Post-rinse (mean±SE)
All	2.72±0.54	4.05±1.63	0.47±0.08	0.5±0.07	0.37±0.31	0.79±0.41
Baseline Plaque Urease	≤ 1.74 units/mg	1.6±0.3 ^a	0.43±0.1	0.53±0.1	0.06±0.03	0.77±0.5
	> 1.74 units/mg	4.4±0.77	0.52±0.13	0.48±0.1	0.91±0.86	0.84±0.8
Baseline Saliva Urease	≤ 0.34 units/mg	5.37±0.3.22	0.19±0.03	0.33±0.06 ^d	0.01±0.004 ^e	0.58±0.55
	> 0.34 units/mg	2.86±0.87	0.76±0.09	0.68±0.09	0.79±0.68 ^e	1.06±0.65
Caries Levels	D ₄ MFS≤1	3.61±0.83 ^b	0.47±0.12	0.45±0.1	0.54±0.49	0.75±0.49
	D ₄ MFS>1	1.64±0.47 ^b	0.48±0.11	0.57±0.09	0.06±0.04	0.87±0.82
Saliva Mutans	< 10 ⁵ CFU/ml	3.47±1.05 ^c	0.45±0.13	0.52±0.13	0.74±0.69	1.05±0.66
	≥10 ⁵ CFU/ml	1.7±0.44 ^c	0.45±0.09	0.49±0.08	0.06±0.04	0.87±0.82

^aPaired t-test: P=0.037 (log-transformation)

^bUn-paired t-test: P=0.015 (log-transformation)

^cUn-paired t-test: P=0.044 (log-transformation)

^dPaired t-test: P=0.018

^eUn-paired t-test: P=0.002 (logit transformation)