Physiologic Determinants of Endothelin Concentrations in Human Saliva

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Background: Salivary endothelin (ET) concentrations have been shown to correlate with disease severity in patients with chronic heart failure (CHF). We undertook the present study to evaluate the stability of salivary ET under different handling conditions to assess its suitability as a biochemical marker in screening, diagnosis, and management of CHF.

Methods: Saliva samples were collected from healthy individuals and/or CHF patients, subjected to different handling conditions, and then stored at −80 °C until assayed by an ELISA for ET.

Results: Salivary ET concentrations showed a time-dependent increase during storage at room temperature. After 72 h of incubation at room temperature, ET increased ~2.8-fold (P = 0.03). Simultaneously, salivary big ET showed a time-dependent 11.2-fold decrease (P < 0.0001). This activity was blocked by an ET-converting enzyme (ECE) inhibitor, suggesting that these changes were attributable to ECE-dependent cleavage of endogenous big ET in saliva. Ex vivo conversion was also observed when samples were stored at 4 °C, but the magnitude of these changes was markedly smaller (P < 0.0001). Posture also affected salivary ET concentrations in CHF patients. With a change from supine to seated rest, salivary ET concentrations increased 1.5- and 1.8-fold after 20 and 40 min, respectively (P = 0.01). With a return to supine rest, salivary ET concentrations returned to baseline concentrations (P = 0.008).

Conclusions: These data suggest that saliva sampling and handling conditions could markedly affect measurement of salivary ET. In particular, care should be taken to minimize ECE-dependent enzymatic conversion of endogenous big ET in saliva.

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Endothelin (ET) is a 21-amino acid peptide that has been found to be a potent vasoconstrictor in various vascular beds (1, 2). In addition, this peptide has mitogenic (3, 4), hypertropic, and profibrotic effects (5) within several organs, which may be of clinical relevance in a variety of disease states. ET is produced primarily by endothelial cells in the vasculature, as well as by several other cell types. Most endogenous release of ET in the vasculature is abluminal, directed toward vascular smooth muscle cells involved in the control of vascular tone (6). Only ~20% of ET is released toward the vascular lumen. As such, plasma concentrations of ET poorly reflect local activation of this peptide, and in situations in which ET concentrations are increased in plasma, marked activation is indicated. Perhaps the most marked activation of the ET system in any disease state occurs in the setting of chronic heart failure (CHF). Patients with established CHF have a two- to threefold increase in plasma concentrations of ET, which correlate with severity of disease (7). Furthermore, plasma ET concentrations provide prognostic information in this setting (8).

Whereas plasma concentrations are increased in patients with CHF, in healthy individuals concentrations are almost undetectable by current assay methodologies. In contrast, salivary ET concentrations have been found to be 3.7-fold higher than plasma concentrations in healthy individuals and to be readily measurable (9). Thus, concentrations of ET in saliva are significantly higher than the lower limits of detection of most assays. Moreover, salivary ET concentrations respond to physiologic stimuli known to alter plasma ET concentrations; specifically, ET concentrations increase with assumption of upright posture in healthy individuals (10). Because saliva is readily

1 Nonstandard abbreviations: ET, endothelin; CHF, chronic heart failure; ECE, endothelin-converting enzyme; ECEi, ECE inhibitor; PBS, phosphate-buffered saline; and RLU, relative light unit(s).
accessible and collectible, it has advantages over blood and urine for biochemical monitoring of chronic conditions.

We recently described increases in saliva ET concentrations in CHF patients compared with healthy individuals (11). The increase was 2.6-fold, comparable to that observed in plasma. Furthermore, saliva ET concentrations increased with worsening disease severity, as judged by New York Heart Association class. We also reported a significant correlation between saliva and plasma ET concentrations in CHF patients. On the basis of these observations, measurement of ET concentrations in saliva may be a simple, noninvasive method to aid in assessment of disease severity in CHF. Although yet to be established, salivary ET may also have utility in tracking response to therapy. There are, however, numerous factors that may affect the measurement of biological substances in saliva, particularly with regard to sample stability and the effects of various external stimuli, such as storage temperature and diurnal variation. Because the influence of these factors is currently unknown, the present study assessed the stability of salivary ET concentrations within various physical settings and physiologic stimuli.

Materials and Methods

Participants
Healthy individuals were 25–50 years of age and gave informed consent to participate in the study. They were on no cardiovascular medications and had no history of diseases likely to alter ET concentrations.

Individuals diagnosed with CHF and attending the Heart Centre at the Alfred Hospital as outpatients consented to participate in the study. All participants were instructed to fast for 1 h before the study to eliminate the confounding effect of food contamination. This study was approved by the Alfred Hospital Human Ethics Committee (CP-98/01).

Sample Collection
Before saliva sample collection, study participants were instructed to rest in the seated position for at least 20 min. A saliva sample was then collected over 2 min in a plastic vial containing a sterile dental cotton roll (Salivette; Sarstedt). Salivary fluid was extracted immediately by centrifugation at 1000g for 5 min and aliquoted into 500-μL tubes for further studies. Samples that were not used immediately were stored at −80 °C. Samples were usually assayed within 1 week of collection, unless otherwise indicated.

Effect of Sample Storage Temperature on Salivary ET
All samples used as storage temperature controls were kept at −80 °C immediately after extraction. Test samples were kept at 4 °C or room temperature (23 °C) for various time points (4–96 h) and then stored at −80 °C for later analysis. Samples were assayed with the Biomedica ELISA for ET. Ten healthy individuals were involved in this study.

Impact of ET-Converting Enzyme Activity in Salivary ET System
Multiple saliva samples from each individual (n = 3) were collected, extracted, pooled, and divided into 2-mL aliquots. One aliquot served as control, and the second aliquot was treated with an agent [ET-converting enzyme inhibitor (ECEi); CGS26303, (S)-2-biphenyl-4-yl-1-((H-tetrazol-5-yl)-ethylamino-methyl phosphonic acid, Novartis] that inhibits the ET-converting enzyme (ECE), which converts the precursor 39-amino acid big ET to the mature 21-amino acid ET. The final concentration of CGS26303 was 50 μmol/L. Each aliquot was then divided into 500-μL volumes and kept at room temperature for 0, 24, 30, or 48 h. At the end of each time period, samples were stored at −80 °C for later analysis.

In a separate experiment, 9 mL of salivary fluid from each of three healthy individuals was divided into 3-mL aliquots (A, B, and C). Aliquot A was treated with phosphate-buffered saline (PBS) only and served as control, rat big ET was added to aliquot B to a final concentration of 10 pmol/mL, and aliquot C was treated with rat big ET and with the ECEi at a final concentration of 50 μmol/L. Treated saliva samples were then aliquoted into five tubes and incubated at room temperature for 0, 4, 8, 24, or 48 h. At the end of each time period, samples were stored at −80 °C for later analysis. All samples were assayed for ET with the in-house-developed ELISA.

Salivary ET Stability During Prolonged Freezing at −80 °C
Twelve saliva samples were collected from healthy individuals, and each sample was assayed twice, immediately after collection and after 5 weeks of storage at −80 °C. The recovery rates were calculated according to the following formula: Recovery rate (%) = 100% × (salivary ET concentration at week 5/salivary ET concentration at day 0). Samples were assayed for ET by the Biomedica ELISA.

Effect of Posture on Salivary ET Concentrations in CHF Patients
Four consecutive saliva samples were collected from each of four CHF patients placed in various postures as follows: (a) after 20 min of supine rest; (b) after 20 min of seated rest; (c) after an additional 20 min sitting; and (d) 20 min after returning to the resting supine position. Saliva was extracted immediately after sample collection at each time point and stored at −80 °C for later analysis. Samples were assayed for ET by the Biomedica ELISA.

Diurnal Variation of Salivary ET Concentrations
Seven saliva samples were collected from five individuals, one sample every 2 h from 0800 in the morning to 2000 in the evening. Samples were stored at −20 °C before extrac-
tion. Salivary fluid was extracted before ELISA. All samples were assayed for ET by the in-house-developed ELISA.

**ET (1–21) MEASUREMENTS**

Salivary ET was measured by a commercially available ELISA (Biomedica). All samples and calibrators were assayed in duplicate. The inter- and intra-assay imprecision (as CV) was 7.6% and 4.5%, respectively. The recovery rate for ET-1 in saliva was >95% with a sample concentration ≥5 pmol/L (12.5 pg/mL). The lower detection limit was 0.05 pmol/L (0.125 pg/mL). Cross-reactivity of the ELISA was 100% to both ET-1 and ET-2, <5% to ET-3, and <1% to big ET. ET-2 and ET-3 are estimated to comprise <20% and 50%, respectively, of the ET-1 concentration in human plasma and similar proportions in human saliva (9). Despite the presence of cross-reactivity to other ET isoforms in saliva samples, the assay measures primarily ET-1. Assays were performed according to the manufacturer’s instructions for measurement of ET in human saliva samples with several modifications as follows: samples were incubated with detection antibody at 4 °C overnight instead of room temperature as instructed; all samples and calibrators were diluted 1:5 before assay; and all samples and calibrators were diluted in protein-based buffer (PB buffer; supplied by the assay manufacturer).

Salivary ET was also measured by an in-house-developed chemiluminescent immunoassay specific for ET-1. This assay is a solid-phase ELISA designed to measure ET-1. The intra- and inter-assay imprecision was determined using high (100 ng/L), medium (25 ng/L), and low (3.13 ng/L) concentrations of controls. Intra-assay imprecision was ≤2.1%, 3.8%, and 3.1%, respectively; inter-assay imprecision was ≤2.1%, 5.9%, and 10%, respectively; and the recovery rate was >95%. The minimum detectable dose of ET-1 was determined by adding 2 SD to the mean relative light units (RLU) for replicates of the zero calibrator and calculating the corresponding concentration; the minimum detectable dose was typically <0.1 ng/L. Cross-reactivity of the ELISA was 100% to ET-1, 15% to ET-2, 1.15% to ET-3, and 0.002% to big ET. In brief, white Immuno plates (MaxiSorp™; Nalge Nunc) were coated with 100 μL of rabbit anti-human ET-1 antibody (Silenus) overnight at 4 °C. After the plate was washed with PBS containing 0.5 mL/L Tween-20 (PBS–Tween 20), it was blocked with 150 μL of Superblock (Pierce) for 1 h at 37 °C and then washed five times in PBS–Tween 20. Calibrators were prepared by serially diluting synthetic ET-1 (American Peptide Company) in sample buffer (1 mL/L Superblock) to final concentrations of 100, 50, 25, 12.5, 6.25, and 3.125 ng/L. Saliva samples were diluted 1:5 (20 μL of sample + 80 μL of diluent) in sample buffer. Calibrators and samples were added to the plate (100 μL/well) with 50 μL/well of mouse anti-human ET-1 antibody (Oncogene) and incubated at 4 °C overnight. After five washes in PBS–TWEEN 20, 100 μL of horseradish peroxidase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) was added to each well and incubated at 37 °C on a shaker (190 rpm). Finally, after five additional washes in PBS–Tween 20, the plate was developed with Supersignal ELISA Femto substrate (100 μL/well; Pierce), read on a microplate Luminometer (Packard Instruments), and the RLU were recorded.

**BIG ET-1 (1–38) MEASUREMENTS**

Salivary big ET-1 (1–38) was measured by a commercially available ELISA (Biomedica). All samples and calibrators were assayed in duplicate. Cross-reactivity of the ELISA was <1% to ET-1/-2/-3 (1–21) and <1% to big ET-2 (1–37). This assay was performed according to the manufacturer’s instructions, with minor modifications as described above for the ET ELISA.

**STATISTICAL ANALYSIS**

Salivary ET and big ET-1 concentrations are expressed as the mean (SD). Statistical analysis was performed using SAS, Ver. 8.2 (SAS Institute Inc.). Tests for trend were performed using Pearson correlation coefficients or with Spearman correlation coefficients when data were non-normally distributed. Where repeat measure data existed, statistical significance was determined using repeated-measures ANOVA. A two-sided P value of 0.05 was considered to be statistically significant.

**Results**

**DEVELOPMENT OF CHEMILUMINESCENT IMMUNOASSAY FOR SALIVARY ET-1**

A typical calibration curve for the in-house chemiluminescent ELISA is shown in Fig. 1. The minimum detectable dose of ET-1 (by definition x + 2 SD of zero calibrator) was <0.1 ng/L. Cross-reactivity of the ELISA was 100% to ET-1, 15% to ET-2, 1.15% to ET-3, and 0.002% to big ET. The interassay imprecision (CV) was 2.1% at 100 ng/L, 5.9% at 25 ng/L, and 10% at 3.13 ng/L.

**EFFECT OF SAMPLE STORAGE TIME AND TEMPERATURE ON SALIVARY ET**

The temperature at which samples are stored appears to influence the ET concentrations in saliva. Salivary ET increased during incubation at room temperature in a time-dependent fashion, from 0.19 (0.05) ng/L at baseline to 4.37 (0.13) ng/L after 96 h incubation, a 23-fold increase ($P < 0.0001$; Fig. 2A). A time-dependent increase in ET concentrations was also observed when the samples were stored at 4 °C, but the magnitude of these changes was smaller [1.53 (0.06) ng/L after 96 h incubation, an eight-fold increase; $P < 0.0001$; Fig. 2B].

**PRESENCE OF BIG ET IN SALIVA**

To investigate the source of the increased salivary ET during storage at room temperature and 4 °C, as shown in Fig. 2, we measured the concentrations of big ET (the 39-amino acid ET precursor) in saliva. We observed a
time-dependent decrease in big ET to a median value of 8.9% (11.2-fold reduction; \( P < 0.0001 \)) of baseline over 72 h at room temperature (Fig. 3A), concomitant with the time-dependent increase in ET concentrations [373.8% of baseline after 40 h at room temperature, a 3.7-fold increase (\( P = 0.0007 \)); and 276.4% of baseline after 72 h at room temperature, a 2.8-fold increase (\( P = 0.03 \)); Fig. 3B]. Conversion was also observed during sample storage at 4 °C, but the magnitude of these changes was markedly smaller (\( P < 0.0001 \)). Big ET in saliva decreased to 64.5% of baseline (1.6-fold reduction; \( P = 0.006 \)) after 72 h (Fig. 4A), with a small attendant increase in ET concentrations (\( P = 0.3321 \); Fig. 4B).

**EFFECT OF ECE INHIBITION ON THE SALIVARY ET SYSTEM**

To further explore the conversion of big ET in human saliva, we used the ECEi (CGS26303) to inhibit endogenous enzyme activity. After incubation for 48 h at room temperature, ET concentrations in saliva increased from 0.85 (0.12) ng/L to 3.34 (0.08) ng/L (\( P < 0.0001 \); Fig. 5A). Addition of ECEi to the saliva samples attenuated the time-dependent increase in ET concentrations [1.54 (0.11) ng/L after 48 h; \( P < 0.0001 \) compared with saliva alone]. When exogenous big ET peptide (rat; 10 pmol/L) was added to the saliva sample (Fig. 5B), the increase in ET concentration was greater than in saliva alone at each time point (\( P = 0.001 \)). Addition of the ECEi in the presence of exogenous big ET attenuated the increase in saliva ET at room temperature: after incubation for 48 h, saliva alone showed a 3.3-fold increase, saliva + big ET showed a 5.67-fold increase, and saliva + big ET + ECEi showed a 3.9-fold increase (\( P < 0.0001 \) for saliva + big ET compared with saliva alone or saliva + big ET + ECEi).

**SALIVARY ET STABILITY DURING PROLONGED FREEZING AT −80 °C**
The effect of prolonged storage (5 weeks) on salivary ET concentrations was assessed. Twelve samples were assessed twice, immediately after collection (day 0) and after 5 weeks of storage at −80 °C. Statistically significant
reductions were observed over 5 weeks of storage ($P = 0.0005$). Approximately 42% of the samples lost >50% of their original activity, 42% lost 40–50% of their original activity, and 17% samples lost <10% activity. Thus, >80% of samples lost ≥10% of the original activity over 5 weeks of storage at −80 °C.

**EFFECT OF POSTURE ON SALIVARY ET CONCENTRATIONS**

The effects of posture on salivary ET concentrations in CHF patients were also investigated in four individuals. Salivary ET concentrations increased to 146.5% of the values for samples collected in the supine position when patients assumed the seated resting position for 20 min ($P = 0.06$). After an additional 20 min of seated rest, salivary ET concentrations were 176.3% of the values for samples collected in the supine position ($P = 0.01$). When patients returned to supine rest for 20 min, salivary ET concentrations returned to concentrations similar to the baseline (supine position) concentrations (94.2%; $P = 0.008$).

**DIURNAL VARIATION OF SALIVARY ET**

The diurnal variations in salivary ET were investigated in five individuals (Fig. 6). There was no clear indication of a particular time of day that salivary ET concentrations were highest. The pattern of production varied among individuals over the day.

**Discussion**

The present study was undertaken to investigate factors that may affect the accuracy of salivary ET values obtained by routine assays for the possible diagnosis and management of patients with suspected and confirmed CHF. We focused on factors that may affect the stability of ET in salivary samples under the handling conditions commonly encountered in clinics, hospital wards, physicians’ offices, and clinical laboratories. These include the influence of temperature, prolonged storage, and patient posture as well as diurnal changes.

Many hormone peptides have been found to be relatively unstable, with the potential for substantial degradation during storage at room temperature (12, 13). In contrast, concentrations of salivary ET in the current study showed a steady increase during incubation at room temperature. The results suggest that there is ECE activity in saliva that continuously converts big ET to ET, producing a time-dependent decrease in big ET and consequent increase in ET. The data also indicated heterogeneity in salivary ET concentrations among individuals. This variability may be attributable to variations in the
availability of big ET or ECE for conversion of ET in saliva. Individual variability in plasma concentrations of ET in apparently healthy individuals has also been reported (14–16). Circulating plasma ET concentrations are significantly increased in African Americans, and the prevalence of hypertension and its complications is also higher in blacks than in whites (15, 16). Plasma ET concentrations both at rest and in response to acute stress are increased in ethnic adolescents (17) and black male adolescents (18). Moreover, plasma ET concentrations are higher in male than in female adolescents (17).

The production of human ET involves expression of the 212-amino acid precursor, preproendothelin, which is cleaved proteolytically to a 39-amino acid residue precursor peptide, big ET (19). Cleavage of big ET at Trp21–Val22 by ECE yields the biologically active ET peptides [ET (1–21), including ET-1, ET-2, and ET-3] and the C-terminal fragment (22–38) (20). ET-2 exhibits the closest structural similarity to ET-1, differing by only two amino acids (91% of homology), whereas ET-3 differs from ET-1 by six amino acids (71% of homology) (21). As mentioned earlier, ET-2 and ET-3 are estimated to account for <20% and 50%, respectively, of the ET-1 in human plasma and for similar proportions in human saliva (9). Therefore, the time-dependent increases in ET in saliva that occur simultaneously with progressive decreases in big ET concentrations suggest the presence of big ET as well as ECE bioactivity in human saliva. This hypothesis was strongly supported by the addition of an ECEi to the saliva samples: CGS26303 successfully inhibited the steady increase of ET in saliva stored at room temperature.

Overall, concentrations of salivary ET increased when samples were incubated at 4 °C for longer periods of time. However, in some samples, a decrease in ET was also observed during the early time points after sampling. This may relate to the balance between ET degradation and the speed of cleavage of big ET to mature ET. ET has a very short half-life in vivo (22), and the presence of proteases and/or other molecules in saliva may also contribute to the degradation of ET in saliva. As expected, it appears from these data that ECE enzyme activity is greater at room temperature than at 4 °C.

It has been reported that postural changes affect plasma and saliva ET concentrations in healthy individuals (10, 23). Because ET concentrations are increased in CHF, we investigated whether salivary ET concentrations respond differently to changes in posture in this setting. Because CHF patients may have difficulty in standing for
demonstrated that salivary ET concentrations change significantly in response to the physical environment. Therefore, these exogenous factors influencing salivary ET concentrations must be considered when using these measurements to monitor diseases such as CHF.

This work was supported by IATIA Ltd. (Melbourne Australia). We wish to acknowledge Dr. Alex Tzanidis for contributions to the design and analysis of the studies.

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